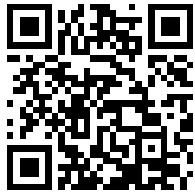

MYCORRHIZAE

PROCEEDINGS OF THE FIRST NORTH AMERICAN CONFERENCE ON MYCORRHIZAE — APRIL 1969 — MISC. PUBLICATION 1189 U.S. DEPARTMENT OF AGRICULTURE — FOREST SERVICE

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LIBRARY OF CONGRESS CATALOG NUMBER 74-609307

EDWARD HACSKAYLO

PRINCIPAL PLANT PHYSIOLOGIST
FOREST PHYSIOLOGY LABORATORY
BRANCH OF TIMBER CULTURE AND ECOLOGY
DIVISION OF TIMBER MANAGEMENT RESEARCH
PLANT INDUSTRY STATION
BELTSVILLE, MARYLAND

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Washington, D.C. 20402 - Price $3
September 1971

Dedication

The authors wish
to dedicate
these proceedings
to

ELIAS MELIN

in commemoration
of a half century of leadership
in research on mycorrhizae.

Foreword

Studies on mycorrhizae in North America have been in progress for over a half century. During the past decade, research efforts here and elsewhere have become particularly intense. Thus the First North American Conference on Mycorrhizae, jointly sponsored by the University of Illinois and the U.S. Department of Agriculture Forest Service, which convened April 1–3, 1969 at the University of Illinois, provided a long overdue opportunity for resident mycorrhiza specialists to meet, present their views, and explore mutual problems.

Participation in the conference was limited to persons who are or were at some time actively engaged in mycorrhiza research or a closely related field. The size of the group was small enough to allow free exchange of ideas between all participants. Invitational papers were presented by plant scientists representing widely diversified specialities but all related to the subject at hand. Each was encouraged to approach his topic from whatever direction he desired as a basis for open discussion after each paper. Short, contributed papers were presented at convenient times during the Conference, and most are included in this volume. At the close of the Conference, A. B. Hatch presented a critique of the contributed papers; this is included in these proceedings.

Drs. E. Hacskaylo and J. W. Gerdemann, co-chairmen of the First Conference, will appoint a steering committee to make preparations for a second conference in 1974.

The efforts of Dr. J. W. Gerdemann and the staff of the Department of Plant Pathology of the University of Illinois, who so successfully hosted the conference, were greatly appreciated. The several distinguished participants who moderated sessions deserve special credit for leading and enriching the discussions. We would like to acknowledge the contribution of the U.S. Department of Agriculture Forest Service in making possible publication of these proceedings. Mrs. D. T. Beslow was of particular assistance to me in direct collaboration in overall planning.

E. Hacskaylo

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Separate No. FS-281

1.

Taxonomy of Ectomycorrhiza-Forming Fungi

Alexander H. Smith

The ability to form mycorrhizae of one type or another is widely distributed throughout the major groups of soil-inhabiting fungi. The fungi producing ectomycorrhizae, however, are primarily Agaricales and gastromycetes which comprise the majority of the higher fleshy fungi. They represent an assemblage of reasonably closely related species which form mycorrhizae with related groups of woody plants, such as pines, aspens, or birches.

History

Higher fungi have been studied taxonomically for the last 150 years or more. They have always been considered difficult to identify, but this is largely due to the way our species concepts developed. In the beginning, investigators identified species by those characteristics obvious to the naked eye—the colors of the fungus and the spore deposit, existence of a ring on the stipe, and relationship of the gills to the stipe. When the microscope came into general use, such features as spore size and shape were emphasized. Now, we observe not only the details of the spore and its ornamentation (if any) but also the details of the hyphae comprising the basidiocarp. As a result, descriptions written by one author have been frequently regarded as incomplete by the next, who then have described additional characteristics about the species to aid in its identification.

Confusion about species described between 1800 and 1900 has been the rule rather than the exception. Specimens were seldom preserved during this period and those which were usually lacked descriptive notes on the fresh material. Thus, during the last 60 years, investigators have been forced to make judgments about previously described species without having access to pertinent data. This confusion will not be dissipated until a neotype for each old European species is established according to a rigid set of rules. If this is not done, each new generation of mycologists will probably use a different name for a given fungus. This is why investigators on this continent often use American names, based on existing type specimens, even if the species is considered by some as synonymous with an earlier-described European species. (*Leccinum aurantiacum* is a European name which has been applied to a number of different species in North America.) *Pholiota adiposa* and *Mycena galericulata* have caused much confusion. In both cases, discrepancies have been great enough to make all reports of the species worthless, especially when they did not indicate exactly which author's concept was followed.

Modern Taxonomic Approaches

The use of the entire basidiocarp in our taxonomy brought to light much more diversity in these fungi than was previously recognized. Thus, an unexpectedly large number of previously undescribed species had to be considered and integrated into our system of classification. The resulting turmoil is especially confusing to those engaged in research areas, such as mycorrhizal formation. An investigator who is moderately well versed in the taxonomy of fleshy fungi may think he knows a certain species—such as *Suillus granulatus*—when actually the species is very complex. Unless investigators record carefully the features of the fresh basidiocarp from which they make their tissue cultures, and then dry the specimen for future use, it may be impossible to determine whether two investigators working with "*Suillus granulatus*", but getting different results, actually had the same variant or even the same species. The data required on an individual collection is often not extensive and is not time-consuming to record. An investigator should note the colors of the pileus, hymenophore, and stipe in both young and old material; presence or absence of veils; color changes in stipe and pileus when sporocarp is cut and again when treated with chemicals; and any peculiar odor or taste. A biologist trying to accurately pinpoint the identity of the organism used in his research can finally consult with a specialist in the systematics of the group concerned.

Identification by a specialist would be much more involved. For instance, in studying hyphal detail, the following questions would be answered: Are hyphal walls thick or thin, colored or colorless, smooth or incrusted? Do the colors of the walls and incrustations differ when mounted in an iodine solution such as Melzer's reagent rather than in water or potassium hydroxide? Do the hyphae break up in the mounting media freeing component cells? Is there secondary septation? Are the hyphal cells tubular, tibiform (enlarged at both ends), keg-shaped, or nearly globose? Are cystidia (end-cells of hyphae) located on the pileus (pileocystidia), stipe (caulocystidia), face of the gills or tubes (pleurocystidia), or edges of the gills or pores (cheilocystidia)? Are hyphae in the hymenophore divergent (extending from a central strand toward the hymenium), convergent (extending inward and downward as seen in a cross section of a gill), or interwoven to parallel?

Also a specialist would study the features of vegetative cultures and the color reactions obtained by applying certain chemicals to the parts of the fresh basidiocarp. KOH, for instance, may give a red or olive reaction. Iron salts may give an olive reaction, a reddish one, or none at all.

The more these fungi are studied, the less reliable tree associations are found to be for taxonomic determinations. But there are many species, both mycorrhiza-forming and nonmycorrhiza-forming, where a constant association has been found to hold true even after ample sampling has been completed.

The Ectomycorrhiza-Forming Fungi

There are many fungi known or thought to be involved in the formation of ectomycorrhizae. In North America, the genus con-

taining the largest number of these species is *Cortinarius*. I predict that approximately 800 species will eventually be recognized on this continent and that very few of these will not be mychorrhiza-formers. *Cortinarius* has one set of species found mainly in deciduous forests and a second set associated with conifers. The genus has been eminently successful in both habitats, but, because it is difficult to cultivate in the laboratory, very little work has been done on the biology of the group.

The presence or absence of gelatinized layers—mainly in the pileus cuticle—is used initially to separate the species of *Cortinarius*. However, in one group (Myxacium), both the pileus and the stipe are slimy. A second group (subgenus *Cortinarius*), consists of species with a dry (fibrillose) pileus; a third group (Telamonia), consists of those having a moist pileus which appears water-soaked when fresh.

Spores of *Cortinarius* are typically rusty brown in deposit and warty-rugulose when observed under high magnification with oil immersion lens. In young basidiocarps, the margin of the pileus is connected to the stipe by a veil of fine fibrils called a cortina.

At the species level, the color of the young gills, the size and shape of the spores, color of pileus, color of outer veil, and odor or taste are used in various combinations for identification. Occasionally the mycelia are colored. The brick red color of *C. rubripes* mycelia allowed Kauffman to trace the fungus from basidiocarp to structures he thought were mycorrhizae of maple, red oak, and bittersweet.

The Boletaceae group is also large. The number of species in North America is unknown, but there are hundreds. Mycorrhizal associations of the genus *Suillus* with conifers have been studied considerably; in fact, all but a few species are limited to conifer associations, a situation quite unlike that found in *Cortinarius*.

The features distinguishing *Suillus* as a genus are a rather interesting combination of cystidial characteristics, gelatinizing hyphae of the pileus cuticle (giving slimy texture to certain species when wet) and tube mouths or pores, which in many species are extended radially producing the appearance of lamellae with well developed cross veins. This extension of tube mouths is termed *boletinoid*. The hymenial cystidia of most species are narrowly clavate to subcylindric. They occur in bunches and secrete copious amounts of a resinous material which, when dried, encrusts the base of the bundle or colors the contents of the cells themselves. The genus as a whole shows some development of a veil-tissue growing out from the margin of the pileus. In some species, this eventually clasps the stipe so firmly that the expansion of the pileus breaks the veil at the pileus margin, leaving a more or less loose ring (annulus) on the stipe.

Individual species may be identified by the presence or absence of a veil, resinous dots or smears on the stipe (clusters of caulocystidia), visicidity of the pileus, and smoothness of the surface. The color of the pileus, spore size, and the color of the spores in deposit are also helpful. Much work remains to be done on the systematics of this genus and the general biology of the species.

The other genus of Boletaceae of special interest from the mycorrhizal point of view is *Leccinum*. The genus, as defined in the

most recent works, is limited to those boletes in which the stipe is punctate to squamulose and in which this ornamentation darkens to dark brown or black as the basidiocarp matures or ages. Species are recognized by spore size, color patterns of the stipe, color of pileus, and color changes in the context of the pileus and the apex of the stipe when the basidiocarp is sectioned. Of importance also are shapes of cells forming stipe ornamentation and the degree to which hyphal contents of the pileus cuticle become emulsified in chloral hydrate solution.

The genus is divided into three sections. The first, *Leccinum,* is distinguished by the sterile membrane continuing from the margin of the pileus and breaking into segments as the pileus expands. The color spectrum is from a dark liver-color to brick-red to orange, yellow or off-white. There were a few species with brown to blackish pilei. A number of these species are associated with aspen, and some, with conifers. Species, such as *L. aurantiacum,* probably can occur with either. Still others associate with white birch.

In the smallest section, *Luteoscabra,* the pileus cuticle is composed of a layer of parenchyma-like cells or of inflated to almost globose hyphal cells which are variously arranged and fairly numerous in the filaments of the cuticle. These species are associated mostly with birch, oak, and hornbeam.

Scabra is a large section, featuring mostly gray to gray-brown to blackish boletes. The filamentous (tubular) cuticular hyphae or filaments have only slightly enlarged cells. In this group, most of the taxonomic problems occur. The best features for the recognition of species, however, are the color changes of the context when cut, spore size, spore shape, size of caulocystidia, formation of secondary septa in the caulocystidia, degree of proliferation of the neck of the cystidium, and degree of pigmentation on the pileus. There are a host of variants around *Leccinum rotundifolieae.* Apparently, this species is the principal one, along with closely similar variants, to form mycorrhizae with dwarf birch. The other species of the section are largely associated with other birches, white birch especially. In fact, *Scabra* may be the principle group of fleshy fungi associated with the birches.

The systematics of the entire genus are at present undergoing intensive study, and a wealth of previously unrecognized species is being described. Thus, the general biological aspects of the group are as yet largely unknown.

The remaining North American boletes do not give as clear a picture of tree associates as do *Suillus* and *Leccinum,* though we know that *Quercus* is a very important associate. If anything, the other genera are more generalized in their associations, and some may not even be involved in the formation of mycorrhizae. The *Boletus edulis* group is troublesome, both as to its systematics and to its possible tree associates if the relationship is not a generalized one.

Since it is assumed by some that *Rhizopogon,* a genus of puffballs in the Hymenogastrales, is distantly related to the boletes, it is appropriate to discuss it here. This genus has at least 150 species in North America which are clearly associated with conifers. *Rhizopogon* must be studied in any serious work dealing with mycorrhiza-forming fungi in the area west of the Great Plains.

A *Rhizopogon* basidiocarp resembles a small potato in shape, but the interior is chambered. The spores are produced on the lining of the chambers. The basidiocarps form in the duff or at the level where duff and mineral soil meet. Naturally, they are difficult to find.

A specialist must know the coloring pattern of the developing basidiocarp in order to identify *Rhizopogon*. It is white at first, then yellow and if exposed to light, finally yellow-brown. Or it may be white at first, gradually darkening to gray-brown to fuscous. Some are a brilliant yellow and remain that way. Color changes such as to red or pink result from bruising. The most accurate way of ascertaining such changes is to cut the basidiocarp in half and apply test solutions to the outer wall. Iron salts give some color change, but they should be applied to the cut surface of the outer wall since a confusing pattern occurs if applied to the surface of some species. The reaction may be blue or green, and if ethanol is applied to the spot, the color may not change at all. Three percent KOH applied to the cut surface of the peridium may give a reddish reaction—or none at all—in some species. In others, there may be a mottled reddish and olive effect, or perhaps only olive. Anatomical details of the peridium, spore size and shape, amyloid reactions and reactions of the organic debris on the hyphae of the peridium to Melzer's reagent can all be obtained from dried material.

Evolutionary Implications

A hypothetical evolutionary series goes from *Rhizopogon* to *Truncocolumella* (a small genus with about three species in our western States) to the Boletaceae in the area of *Suillus*, to *Fuscoboletinus* (a small genus of boleti with less than a dozen species known in North America), which has reddish to chocolate-colored or violaceous drab spores in contrast to the olive, yellowish or cinnamon spores of *Suillus*. Finally, our evolutionary series goes to *Gomphidius* and *Chroogomphus*, true gilled fungi having prominent, thick lamellae. The two genera of the Gomphidiaceae clearly appear related to *Fuscoboletinus* by the color of the spore deposit and the almost lamellate configuration of the hymenophore. Also, in some species of *Gomphidius*, the elongate cystidia occur in bunches on the stipe and are chemically reactive much like those on many *Suillus* species. There are similarities in spore shape, but not in spore size.

This entire hypothetical evolutionary line with *Rhizopogon* at one end and *Gomphidius* at the other is interesting because of the morphological changes of a gastromycete type of basidiocarp to a bolete type and then a mushroom-type with gills. And at each level in the series, the species are mycorrhiza-formers (assumed mostly on the basis of field studies) with conifers. In the Pacific Northwest, all three major evolutionary levels are present in greater numbers than anywhere else in the world. The *Rhizopogon* explosion created over a hundred species, ten times that known from any other areas. *Suillus* and Gomphidiaceae have also reached a peak in their development. There are more species of the latter in this region than in any other region in the world. It may not be a coincidence that west of the Great Plains, we also have a major area for

evolution of species of conifers. This suggests that evolution of a related group of mycorrhiza-forming species on conifers has proceeded hand in hand with the evolution of the conifer species themselves.

A second hypothetical evolutionary line extends from the unbranched clavarioid fungi through the large branched corals (*Ramaria* sensu Corner) to *Cantharellus* and *Gomphus* to the Hygrophoraceae to *Clitocybe* and related gilled fungi in the Agaricales. Little can be positively stated about tree associates of the species in this group. The species are about as abundant in deciduous as in conifer forests, and field observations have not clearly connected any substantial number of fungal species with single species of trees or even with certain genera.

Hygrophorus is better known in this respect than any of the other genera, but even here much remains to be done. Also, scattered through many of these genera one finds more species that might not be mycorrhiza formers than in the series previously discussed. Our knowledge is very sketchy about such genera such as *Ramaria* sensu Corner, for which there is yet no satisfactory treatment of the American species. One thing is clear, however: the data for this group do not point to a conclusion supporting parallel evolution as in the series previously discussed. *Ramaria*, for instance, is well represented in numbers of species in the deciduous forests of our Southeastern States and is equally abundant in the conifer forests of the Pacific Northwest. *Cantharellus* and related genera follow the same pattern as *Hygrophorus* and *Clitocybe*. A critical study of the mycorrhiza-forming ability of species in this series would be most interesting as compared to the bolete series. Some theories on the evolution of the higher fungi hold that the *Clavaria-Ramaria-Cantharellus-Hygrophorus-Clitocybe* series is in general made up of more primitive types than the fungi of the bolete series.

Host-Species Relationships

What about the pattern of host-species relationships within a large genus such as *Suillus* in the boletes? Are all the species, for instance those restricted to white pine (*Pinus strobus*), more closely related than to other *Suillus* species associated with other conifers? *S. americanus, S. pictus, S. placidus* are three common species well known to produce basidiocarps only in close association with *Pinus strobus*. In the most recent American classification of the *Suilli, S. pictus* is found in the section *Boletinus*. The other two are found in section *Suillus*. *S. americanus* has a cottony marginal "veil," whereas in *S. placidus*, the margin of the pileus is naked; no veil material is present at any stage of development. This serves to place both species in separate groups (series). In other words, within the genus, the species clearly associated with only white pine are not so similar that they are all classified in the same subgroup, and they do not form what is known as a species-complex. Testing the opposite idea by taking a species such as *S. pictus* which is constantly associated with white pine, and basing considerations of relationships on the similarities of their basidiocarps, we find

that *S. pictus* is quite similar to *S. cavipes*. It is also similar to *S. lakei* which forms mycorrhizae with Douglas-fir. It is this latter pattern of association which presently appears to be encountered most frequently. Thus, in a given evolutionary line within a genus such as *Suillus*, there was selection by the environment—in this case, different species of conifers occurring in a given region—and there was some factor which enabled certain variants of the original stock to establish a relationship with a different tree species than that of the parent stock, and to survive in competition in a sense, with the parent stock. In speculating along such lines, however, we must also keep in mind that, while such a process as just postulated might have been going on, we also had (and still have) many species which form mycorrhizae on a number of conifer species (*Pinus*, for instance) so that in any genus like *Suillus*, a number of patterns of evolution might be going on simultaneously. *Suillus granulatus*, for instance, is a complex of variants not necessarily limited to any one species of pine.

There are additional major groups of fungi of interest to the student of mycorrhizae. *Russula* and *Lactarius* are two genera which together form the family Russulaceae and contain over 350 species in North America. *Tricholoma* has over 100, *Amanita* around 75, *Rhodophyllus* about 300, *Clitocybe* about 150, and *Hebeloma* about 50. Counting the number of species in the *Cortinarius* group, the bolete series and the *Clavaria-Cantharellus* series, not already included above, there are about 2,100 species of fungi which may be mycorrhiza-formers that belong to the mycorrhizal group (figures based on my unpublished manuscripts). The actual number is probably much higher as many genera have not been considered. How much physiological diversity is represented in a group of this size? If there is a parallel between physiological and morphological diversity, certainly the group is worth further study.

We should not drop our survey of mycorrhiza-forming species without a word on the genera usually not considerd to belong among these. First of all, in large genera like *Cortinarius*, there are some species which form mycorrhizae with numerous host species, or perhaps do not form mycorrhizae at all. Probably there are some of this latter type in all the previously named genera. However, there are large genera which appear to be composed mostly of non-mycorrhiza-forming species: *Psathyrella, Coprinus, Mycena, Galerina, Marasmius*, and *Pholiota*, to name some. However, there are species in these genera which might be involved in the formation of mycorrhizae, such as *Mycena pura*. In North America, *Psathyrella* numbers about 400 species, *Pholiota*, 200; *Mycena*, about 200; and *Coprinus*, over 100. *Galerina* has about 200 species worldwide. The total for the non-formers would be over a thousand species for North America alone. These figures are approximations and serve only to indicate that both groups are very large. It is evident that one finds the same degree of diversity relative to the formation of mycorrhizae in each group, but in reverse proportions. From the standpoint of the natural relationships, the fungi classed by groups as nonmycorrhiza-formers do not connect to those groups in which most of the species form mycorrhizae. This is evident in the Singer system of classification, which is our closest approximation to an overall natural classification of the Agaricales.

Conclusion

The study of mycorrhizae is just in its infancy. In the accompanying, short bibliography are listed modern monographs of interest to those involved with North American fungi. The species concepts in all these works are consistent. These concepts seem narrow to most people, but to a considerable extent, this is because of the way index characters are used. Often a difference in one index character appears to be the major distinction between two populations being interpreted as different species. However, final judgment on the acceptability of what is a broad or narrow concept should rest on increased knowledge of the biology of the populations. In this light, many of our presently delimited "narrow" species may actually encompass a high degree of biological diversity. The goal in our system of classification is an arrangement which approximates the actual relationship by descent of the present day populations. The value of such a natural system to a mycorrhiza investigator, is that he can expect closely related species to have rather similar biochemical processes producing the same or closely related compounds, and such a classification furnishes him with a guide to what species to test for a particular synthesis. Thus, the truly natural system of classification has aspects of practicality as well as being the ultimate in elegance to the systematist.

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2.

Fungi That Form the Vesicular-Arbuscular Type of Endomycorrhiza

J. W. Gerdemann

In nature, the mycorrhizal condition is the rule, the nonmycorrhizal condition, the exception. The roots of both cultivated and uncultivated plants are usually infected with mycorrhizal fungi. The morphology of these infected roots, the mycorrhizae, varies considerably from one group of plants to another, and each type of mycorrhiza has a characteristic group of fungi capable of producing it.

Mycorrhizae are generally divided into two main groups—the ectomycorrhizae and the endomycorrhizae. The ectomycorrhizal type, while it has been studied more than any other kind, is not the most common. It is restricted almost entirely to trees in a relatively small number of families. Members of the Pinaceae, Fagaceae, and Betulaceae are nearly always ectomycorrhizal. In addition, several other Angiosperm families, the Salicaceae, Juglandaceae, Tiliaceae, Myrtaceae, and Caesalpinaceae have both ectomycorrhizal and endomycorrhizal species, and occasionally both types occur on the same plant. Under some conditions, ectendomycorrhizae develop on some ectotrophic species, and it may be considered a modification of the ectomycorrhizal type.

Endomycorrhizae are divided into two subgroups: those produced by septate fungi and those produced by nonseptate fungi. Those formed by septate fungi occur primarily on Orchidaceae, Gentianaceae, and Ericales. Endomycorrhizae formed by nonseptate fungi, often referred to as phycomycetous or vesicular-arbuscular (VA) mycorrhizae, occur on more plant species than any other type. This fact is not generally recognized, probably because this kind of infection produces very little, if any, change in external root morphology. As a consequence, plants with VA mycorrhizae are very often assumed to be nonmycorrhizal. Vesicular-arbuscular mycorrhizae are so common that it is far easier to list the families in which they are not known to occur than to list the families in which they occur. If one excludes ectomycorrhizal species, endomycorrhizal species with septate endophytes, and a few families which may be nonmycorrhizal, the remaining plant groups have VA mycorrhizae (Gerdemann, 1968). It occurs on Bryophytes, Pteridophytes, Gymnosperms, and Angiosperms throughout the world.

Since there are very few plant associations that do not contain some species that normally have VA mycorrhizae, it follows that the fungi that produce this kind of infection must be present in nearly all soils.

Identity of the Endophytes

There is now conclusive evidence that vesicular-arbuscular mycorrhizae are formed by *Endogone* species. Because of the difficulty in isolating these endophytes, their identity long remained a mystery. Many investigators attempted to isolate the fungi from mycorrhizae; however, in nearly every instance they obtained common soil and root inhabiting fungi rather than the true endophyte. A few investigators who isolated species of *Fusarium, Rhizoctonia,* or *Pythium* believed that they had isolated the endophyte, but most recognized the fact that they had obtained a root pathogen or a saprophytic fungus closely associated with the root. Only one investigator may have isolated the endophytes. Barrett (1961) using a complex hemp seed baiting technique, obtained a number of isolates from roots that closely resembled the endophyte, and he was able to synthesize mycorrhizae with his cultures. I used Barrett's method to obtain two isolates that appeared nearly identical with his; however, I was unable to obtain mycorrhizal infection with them. Most of our knowledge about VA mycorrhizae has not been obtained with pure cultures.

Dangeard (1900) was the first to name a VA fungus. He described a typical VA mycorrhiza on poplar and named the endophyte *Rhizophagus populinus*. However, he had no knowledge of its proper relationship. Peyronel (1923, 1924, 1937) was the first to suggest that the VA fungi were *Endogone* species. By tracing hyphae from *Endogone* sporocarps to mycorrhizal roots, he obtained the first evidence for the identity of the endophytes. Later, Butler (1939) noted the resemblance of vesicles of VA fungi to chlamydospores of *Endogone,* and he concluded that the fungi were probably *Endogone* species that had generally lost the ability to form sporocarps.

Mosse (1953, 1956) was the first to demonstrate experimentally that *Endogone* species can produce VA mycorrhizae. Using sporocarps or spores of an unnamed *Endogone* species that she found attached to mycorrhizal strawberry roots as inoculum, she synthesized typical VA mycorrhizae. The species that she discovered was later named *E. mosseae* in her honor (Nicolson and Gerdemann, 1968).

Prior to 1953, *Endogone* species were believed to be quite rare, and all of the collections of sporocarps had been found mainly in forest soils or in sphagnum bogs.

The wet-sieving and decanting method, commonly used to obtain nematodes from soil, was adapted to assay soils for *Endogone* spores (Gerdemann, 1955, 1961; Gerdemann and Nicolson, 1963; Mason, 1964; Nicolson and Gerdemann, 1968; Mosse and Bowen, 1968a and b). Most of the spores found using this method are borne free in the soil rather than in sporocarps. These extremely large spores have been found in abundance in many parts of the world. *Endogone* spores are particularly common in cultivated soils, and although forest soils have not been extensively examined, preliminary studies suggest that the spores may be somewhat less abundant in forests. This does not necessarily mean that the fungi are less prevalent. It is possible that fewer spores are produced or

that the *Endogone* species most common in forests produce small spores which are difficult to detect. The collection techniques used by Gilmore (1968) may prove to be more useful for obtaining *Endogone* species from forest soils than wet-sieving and decanting.

Spores extracted from soil by wet-sieving and decanting have been used to establish "pot cultures." Such a culture consists of a single *Endogone* species maintained on the roots of living plants grown in partially sterilized soil. A soil sample is mixed with water and the heavier particles are allowed to settle for a few seconds. The suspension is then decanted through a sieve fine enough to remove the larger particles but coarse enough to allow the desired spores to pass through. The suspension that passes through this sieve is retained and mixed again. The heavier particles are again allowed to settle, and the suspension is decanted through a sieve fine enough to retain the desired spores. The organic debris and spores retained on this sieve are thoroughly washed and examined under a stereoscopic microscope. *Endogone* spores are then separated from the debris with a flattened needle. By carefully selecting similar spores and using them as inoculum, "pot cultures" of a number of *Endogone* species have been established.

Ohms (1957) described a density gradient flotation system for separating spores from the debris retained on sieves, and more recently, Mosse and Jones (1968) devised a method to separate spores from the organic fraction by differential sedimentation on gelatin columns.

Endogone cultures have also been obtained by transplanting field grown plants to pots of sterilized soil or inoculating plants growing in pots of sterilized soil with field collected mycorrhizae (Gilmore, 1968). Mycorrhizal development and *Endogone* spore production are generally enhanced in sterilized soil, and "pot cultures" of individual *Endogone* species can be established with selected spores picked from the mycorrhizae with tweezers.

Some soils may contain only one *Endogone* species. I obtained a "pot culture" of *Endogone fasciculata* by planting maize in a subsoil that had been stored dry in the greenhouse for one and one half years (Gerdemann, 1964, 1965).

"Pot cultures" of individual *Endogone* species have been used to determine the life cycles of species, their host ranges, and their effect on nutrient absorption and plant growth.

Host Ranges

Mycorrhizal *Endogone* species have extremely wide host ranges. There are very few restrictions on their ability to infect roots and produce mycorrhizae. A single *Endogone* species can produce mycorrhizae on such diverse crops as maize, red clover, soybean, onion, and strawberry (Gerdemann, 1961) or maize and tuliptree (Gerdemann, 1965). No definite limitations have been reported within the species that normally have VA mycorrhizae. We have failed, however, to obtain mycorrhizal infection with *E. mosseae* on pine, which is normally ectomycorrhizal, or on cabbage which belongs to a family believed to be nonmycorrhizal.

The Endogonaceae

The family Endogonaceae contains one large genus, *Endogone*, and two smaller genera, *Sclerocystis* with 3 species and *Glaziella* with only one species. Link (1809) described the first *Endogone* species, and by 1935, the genus had grown to 20 species (Zycha, 1935). However, specimens were rarely collected, and several species known only from the type material were inadequately described. For many years the relationship of the family to other fungi was uncertain. Because of the superficial resemblance of their fruiting bodies to sporocarps produced by species of Tuberales, the Endogonaceae were generally considered to be ascomycetes.

Bucholtz (1912) first described sexual reproduction in this group in detail, and as a result of his work, the family was placed in the Phycomycetes in the order Mucorales. Our knowledge of the family and the genus *Endogone* is still extremely poor. Zygosporic, chlamydosporic, and sporangial species have been included in *Endogone* with little evidence that the three types are related. They were grouped together because all three types produce sporocarps and the zygospores and chlamydospores are somewhat similar.

Sporocarps containing both chlamydospores and zygospores have been found for only two species: *E. fasciculata* (Thaxter, 1922) and *E. microcarpa* (Godfrey, 1957). In the case of *E. fasciculata*, it is likely that the specimen containing two types of spores consists of a mixture of a zygosporic and a chlamydosporic species (Gerdemann, 1965). There is some evidence that the sporangial species may belong to the Mortierellaceae (Walker, 1923). Kanouse(1936) isolated a zygosporic species, and in culture it produced chlamydospores, zygospores, and sporangia. The sporangia of this isolate possessed columellae, and since these structures are lacking in the sporangial *Endogone* species, she assigned them to a new genus, *Modicella*, which she placed in the Mortierellaceae. I believe this work should be confirmed before the revision is accepted.

The recent discovery that some species produce chlamydospores both in sporocarps (endocarpic) and free in the soil (ectocarpic) has made it necessary to modify the genus to include nonsporocarpic species (Nicolson and Gerdemann, 1968). In cultivated soils, ectocarpic spores are much more common than endocarpic, and it now appears that there are a number of species that either rarely form sporocarps or else fail to produce them at all.

Yet another group of "*Endogone*--like" fungi recently has been discovered. Their spores, which are often extremely large, are, as far as known, always ectocarpic. In many respects these spores resemble zygospores; however, they form in a distinctly different way from the zygospores produced by sporocarpic species, and it may be necessary to eventually transfer these fungi to a new genus (Nicolson and Gerdemann, 1968).

At present, the following four types of species are included in *Endogone:*

I.—Zygospores borne in sporocarps. Spores form in two distinctly different ways. Intermediate stages relate them. (A) Two equal gametangia unite at their tips, and a zygospore buds from the point of union. (B) A small gametangium unites with a larger game-

tangium, and the zygospore grows out from the tip of the larger gametangium. Germination of spores has not been observed. Evidence for a mycorrhizal relationship exists for only one species. *E. lactiflua* has been found closely associated with ectomycorrhizae of pine in Italy (Fassi, 1965).

II.—Sporangia borne in sporocarps. There is no evidence that species in this group are mycorrhizal.

III.—Chlamydospores borne in sporocarps and free in the soil. Spores germinate by producing a germ tube from the subtending hypha. Smaller spores sometimes form within the chlamydospores. A number of species in this group form vesicular-arbuscular mycorrhizae.

IV.—Zygospores or azygospores borne free in the soil. Sporocarps are unknown. Spores are produced terminally on a single suspensor-like bulbous structure. A hypha often extends from the bulbous structure to the spore. Spores germinate by producing germ tubes directly through the spore wall. All known species produce endomycorrhizae with arbuscules. Vesicles within roots are unknown. Distinctive vesicle-like structures form on hyphae in soil. The "white-reticulate" species described by Mosse and Bowen (1968) which lacks a distinct bulbous attachment probably belongs to this type. Also, their "honey-coloured sessile" species, (Spore type 6 [Gerdemann and Nicolson, 1963]) with spores born laterally from a hypha which has a thin-walled vesicle at its tip, may be related to this group.

Endogone Species Known to be Mycorrhizal

The following named and unnamed species have been shown to be mycorrhizal in inoculation experiments. Detailed descriptions and illustrations can be found in the listed references.

Key To Species:

1. Chlamydospores formed. Spore wall-thickening extends down subtending hyph. Germ tube emerges from subtending hypha. Form mycorrhizae with arbuscules and vesicles ..... (2)
1. Zygospores or azygospores formed. Spore wall-thickening does not extend down subtending hypha. Very narrow pore at base of spore. Germ tubes emerge directly through spore wall. Form mycorrhizae with arbuscules. Vesicles within mycorrhizae not reported except in that formed by one unnamed species, "spore type 6". Distinctive vesicles in soil ..... (6)
2. Spores always under 100μ, generally ectocarpic. Sporocarps rare, without peridium ..... *E. fasciculata*
2. Spores generally over 100μ ..... (3)
3. Base of spore funnel-shaped ..... (4)
3. Base of spore not funnel-shaped ..... (5)
4. Funnel short. Subtending hypha occluded by extension of inner spore wall, Spores both ectocarpic and endocarpic. Small sporocarps with thin peridium ..... *E. mosseae*

4. Funnel long. Normal septum at funnel base ..... Unnamed species *Funnel-shaped spores* (Moses and Bowen, 1968 a,b)

5. Spore wall thick and brown, thin outer wall lacking. Spores 91-318μ ..... *E. macrocarpa* var. *geospora*

5. Spores as above, except smaller 84-134μ. Sporocarps rare, without peridium ..... Considered a small-spored form of *E. macrocarpa* var. *geospora*

5. Spores yellow, loosely enclosed in a thin, outer wall. Sporocarps unknown ..... *E. macrocarpa* var. *caledonia*

6. Spores borne laterally from a hypha that terminates in a vesicle ..... Unnamed species *Spore type 6* (Gerdemann & Nicolson, 1963) *Honey-coloured sessile spores* (Mosse & Bowen, 1968 a,b)

6. Spores terminal ..... (7)

7. Subtending hyphae simple ..... Unnamed species. *White reticulate spores* (Mosse & Bowen, 1968 a,b)

7. Subtending hyphae bulbous ..... (8)

8. Spores usually over 250μ, greenish yellow. Soil-borne vesicles echinulate, formed in clusters ..... *E. gigantea*

8. Spores under 250μ diam. ..... (9)

9. Spores brown. Soil-borne vesicles smooth, formed in clusters ..... *E. heterogama*

9. Spores cream or light greenish yellow. Soil-borne vesicles warty, produced singly ..... *E. calospora*

Endogone fasciculata Thaxter. This species produces relatively small, thick-walled chlamydospores that are generally borne in loose clusters. Compact sporocarps are rare. It is probably a common species; however, because of its small spores, it can be easily overlooked. The mycorrhizae it produces are characterized by an abundance of thick-walled vesicles that may damage the root cortex. These vesicles are identical to the chlamydospores produced in the soil.

Common names: Probably Cultures E3, E5, and E6 (Gilmore, 1968).

Other references: Thaxter, 1922; Godfrey, 1957; Dowding, 1959; Nicolson, 1959; Gerdemann, 1965; Gray and Gerdemann, 1967.

Endogone mosseae Nicolson and Gerdemann. The chlamydospores formed by this species are produced either free in the soil (ectocarpic) or in small sporocarps (endocarpic). One form found in Germany and the USA (Illinois) also produces chlamydospores within roots. In comparison with the chlamydospores, most vesicles within the mycorrhizae remain relatively small and thin-walled.

This species has been found in England, Scotland, Germany, the U.S.A. (Illinois and California), Australia, and New Zealand.

Common names: Sporokarpienmycorrhiza (Meloh, 1963); Spore type 1 (Gerdemann and Nicolson, 1963); yellow-spored species (Stevenson, 1964); yellow vacuolate spores (Mosse and Bowen, 1968 a,b); Culture E2 (Gilmore, 1968).

Other references: Mosse, 1953, 1956, 1957, 1959a, 1959b, 1962; Mason, 1964; Gerdemann, 1961, 1964; Holevas, 1966; Daft and Nicolson, 1966; Murdoch et al, 1967; Nicolson and Gerdemann, 1968; Gray and Gerdemann, 1969.

Endogone sp. (unnamed). Funnel-shaped spores (Mosse and Bowen, 1968 a,b). The chlamydospores of this species have a long and very striking funnel-shaped spore base. It has been found only in Australia.

Endogone macrocarpa var. *geospora* Nicolson and Gerdemann. This variety produces brown, thick-walled spores free in the soil and within roots. Sporocarps have not been observed (Nicolson and Gerdemann, 1968). It has been found in Scotland.

Common names: Spore type 3 (Nicolson and Gerdemann, 1968). The red-brown laminate spores described by Mosse and Bowen (1968 a,b) resemble those formed by this species. However, the "red-brown laminate species" produces small sporocarps containing one or two spores. Therefore, it is probably a distinct species.

Other references: Daft and Nicolson, 1966.

Endogone macrocarpa var. *geospora* Nicolson and Gerdemann *small-spored form.* This apparently differs from the typical form only in its smaller spore size. It has been found in Illinois and in the delta region of Mississippi. One sporocarp was found in a pot culture. It is slightly less than 1 mm in diameter and contains perhaps 40 or more chlamydospores without an enclosing peridium. It is quite different from the sporocarps Mosse and Bowen (1968a) reported for their "red-brown laminated spore" species.

Endogone macrocarpa var. *caledonia* Nicolson and Gerdemann This variety produces chlamydospores free in the soil. Sporocarps have not been found. The light yellow spores are enclosed in a thin layer which can be easily removed. It has been reported only from Scotland.

Common names: Spore type 2 (Gerdemann and Nicolson, 1963).

Other references: Daft and Nicolson, 1966; Nicolson and Gerdemann, 1968.

Endogone sp. (?) (Unnamed). Spore type 6 (Gerdemann and Nicolson, 1963). *Honey-coloured sessile spores* (Mosse and Bowen, 1968 a,b). The spores of this species, which are probably zygospores or azygospores, are produced laterally on a hypha which terminates in a large, thin-walled vesicle. Sporocarps have not been observed. Although this species may be related to *Endogone*, it is doubtful if it should be included in this genus. It has been found in Scotland, Australia, and New Zealand.

Endogone sp. (Unnamed). *White reticulate spores* (Mosse and Bowen, 1968 a,b). The spores of this species are probably zygospores or azygospores. Although it lacks the bulbous subtending hypha found in *E. gigantea, E. heterogama,* and *E. calospora,* its method of germination and general appearance would seem to re-

late it to this group of species. It has been found in England, Australia, and New Zealand.

Endogone gigantea Nicolson and Gerdemann. The striking greenish-yellow spores of this species are probably the largest produced by any fungus. These spores, which are probably zygospores or azygospores, have a single bulbous suspensor with a slender hypha extending from it to the spore. It also produces clusters of echinulate vesicles in the soil. Sporocarps are unknown. It produces endomycorrhizae with thick-stalked arbuscules. Vesicles within mycorrhizae are unknown. It has been found in Illinois, Indiana, and South Dakota.

Common names: B spores (zygospores) and C spores (vesicles) (Gerdemann, 1955); Sporenmycorrhiza (Meloh, 1963); Mosse and Bowen, (1968a) list this species as occurring in Australia and New Zealand and refer to it as "Bulbous, vacuolate spores." Their descrition, however, suggests that the fungus they found may not be *E. gigantea.*

Other references: Ohms, 1956; Clark, 1969.

Endogone heterogama Nicolson and Gerdemann. This species is easily distinguished from *E. gigantea* by its much smaller, brown spores. The soil-borne vesicles are smooth and formed in clusters. It has been found only in Illinois.

References: (Nicolson and Gerdemann, 1968).

Endogone calospora Nicolson and Gerdemann. In this species the slender tube that extends from the bulbous suspensor is often incompletely developed or lacking. The soil-borne vesicles are warty or irregular in shape and are produced singly. It has been found in Scotland, England, New Zealand and U.S.A. (Illinois).

Common names: Spore type 5 (Gerdemann and Nicolson, 1963); Bulbous reticulate spores (Mosse and Bowen, 1968 a,b).

Other references: Nicholson and Gerdemann, 1968.

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3.

Mycorrhiza-Forming Ascomycetes [1]

James M. Trappe

History

The hypogeous Ascomycetes have figured prominently in the prehistory and history of mycorrhizal studies. Vittadini (1831), in his first epic work on these fungi, noted that species of *Elaphomyces* and Tuberales occur among rootlets of certain vascular plants. Tulasne and Tulasne (1841) went a step further in their studies of *Elaphomyces*. They observed that ascocarps of some species are encased by proliferated tree root tips (figs. 1 and 2) whose surfaces are enveloped by outer ascocarp hyphae. They interpreted this phenomenon as parasitism of the roots by *Elaphomyces*. Vittadini (1842) confirmed the Tulasne observations but disputed their conclusions. He pointed out that the rootlets multiply unharmed around the ascocarps. In perhaps the first perception of

[1] These studies were supported in part by grants from the American Philosophical Society and the Society of The Sigma Xi.

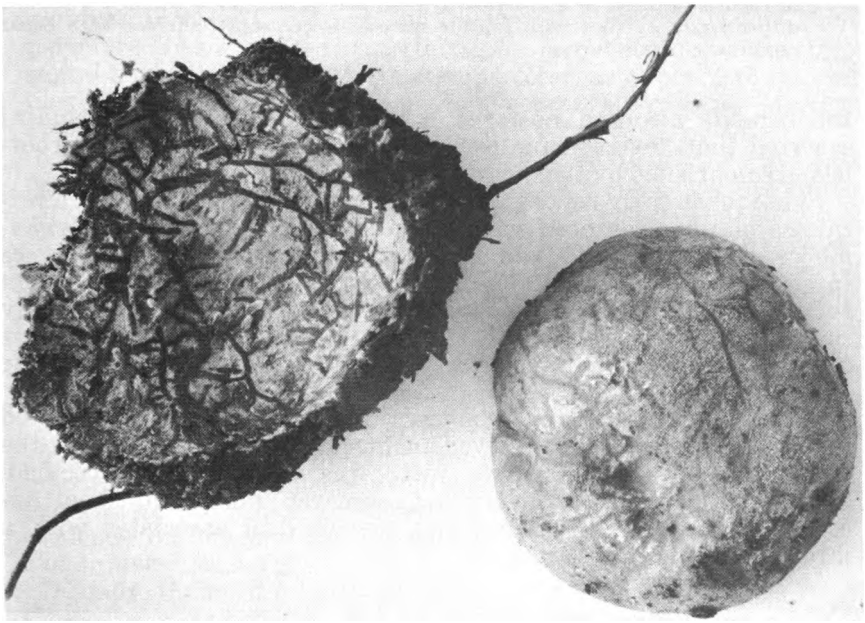

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Figure 1.—Ascocarp of *Elaphomyces granulatus* Fr. with outer crust of hyphae and proliferated ectomycorrhizae of *Abies amabilis* removed (piece of crust at left). Scale in mm.

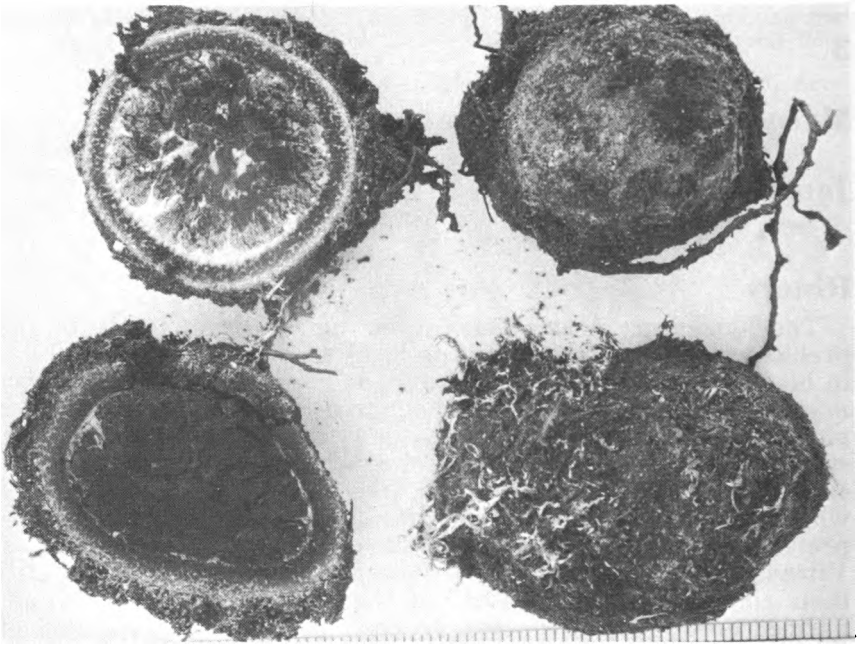

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Figure 2.—Ascocarps of *Elaphomyces muricatus* Fr. Left, cross sections showing dark, powdery spores surrounded by a thick, marbled peridium which in turn is encased in a crust of hyphae and proliferated ectomycorrhizae; upper right, surface with crust removed; lower right, surface with crust remaining. Scale in mm.

the benefits afforded hosts of mycorrhizal infections, Vittadini asserted that "extra omne dubium"—beyond all doubt—the rootlets are nourished by their enveloping *Elaphomyces* hyphae.

Subsequent early reports of what are now regarded as mycorrhizal associations centered on "parasitism" of tree roots by *Elaphomyces*. Boudier (1876) and Reess (1880) described *Elaphomyces* infections of roots in detail sufficient to confirm that they had observed ectomycorrhizae. Gibelli (1882) described and beautifully illustrated mycorrhizae with the mantle structure distinctive for certain *Elaphomyces* species as well as *Cenococcum graniforme* (Sow.) Ferd. & Winge.

When he coined the term "mycorrhiza," Frank (1885) indicated that many fungi were involved in mycorrhiza formation but the Ascomycete, *Tuber aestivum* Vitt., was the only one specified. Since that landmark in mycorrhiza research, many additional Ascomycetes have been proposed as mycorrhizal associates with a large variety of vascular plants.

Evidence of Mycorrhizal Habit of Ascomycetes

Most of the Ascomycetes that have been proposed as mycorrhiza formers fruit hypogeously only near ectomycorrhizae of vascular plants. This obligate habit, the nearly universal difficulty of growing them in pure culture, and the complete absence of any sign of

pathogenicity strongly indicate their symbiotic character. Additional evidence is now available from anatomical and cultural studies.

The observations of Boudier (1876) and Reess (1880) provided the first evidence of anatomical connection between mycorrhizae and specific fungi, in these cases *Elaphomyces* spp. The detailed research of Reess and Fisch (1887) on the association of *Pinus sylvestris* [2] with *Elaphomyces granulatus* Fr. and *E. variegatus* Vitt. has strongly enforced this evidence. Dominik (1961) added further anatomical evidence in describing *Picea abies/E. granulatus* mycorrhizae, as did Fontana and Centrella (1967) with *Fagus* and *Castanea/E. muricatus* Fr. The proliferated mycorrhizae that grow appressed against the ascocarps of *Elaphomyces* spp. permit rather straightforward confirmation of their anatomical interrelationship.

In the case of Tuberales, the mycorrhizae are not usually so closely appressed to the ascocarps. A mycelial connection between mycorrhiza and ascocarp is often not detectable, although Dangeard (1894) observed abundant rhizomorphic connections between *Castanea* mycorrhizae and *Tuber* ascocarps. More conclusive evidence has recently been reported by Italian researchers: The mantles of mycorrhizae associated with various species of Tuberales strikingly reproduce the distinctive peridial anatomy of their respective fungal associates (Fassi and de Vecchi, 1963; Fassi and Fontana, 1967; Fontana and Centrella, 1967). Scannerini and Palenzona (1967) have further refined this work by electron microscopy of mycorrhizae associated with ascocarps of *Tuber borchii* Vitt. (=*T. albidum* Pico ex Cer.) and having mantles similar to the *Tuber* peridium. The hyphae of the Hartig net proved to have the simple septal pores that characterize Ascomycetes, as opposed to the complex dolipores typical of Basidiomycetes (Moore and McAlear, 1962).

Experimental confirmation of mycorrhiza formation by Ascomycetes has been limited by the general lack of success in growing candidate species in pure culture. Sappa (1940) reported mycorrhiza formation on *Quercus* roots grown with purported *Tuber magnatum* Vitt. mycelium. Unfortunately, his isolation technique left ample opportunity for contamination by other mycorrhizal fungi. Hawker (1954) mentions aseptic synthesis of mycorrhizae of *Fagus* grown with isolates from ascocarps of *Tuber excavatum* Vitt. but gives no details.

The intensive research on isolation of *Tuber* species, now in progress at the University of Torino, Italy (Fontana and Palenzona, 1969), will, we hope, open up the possibility of confirming their mycorrhizal habit in aseptic culture. Meanwhile, well-designed pot experiments by Fassi and Fontana (1967) have provided strong evidence on mycorrhiza formation between *Pinus strobus* and *Tuber maculatum* Vitt.

Finally, the most intensively studied of all the mycorrhizal fungi, *Cenococcum graniforme* (Sow.) Ferd. & Winge deserves mention. Through electron microscopy, Trappe and Addison (unpublished data; see fig. 3) have confirmed that the septal pores of *C. grani-*

[2] Latin names of host plants are acording to Little (1953) for American species and Kelsey and Dayton (1942) for others.

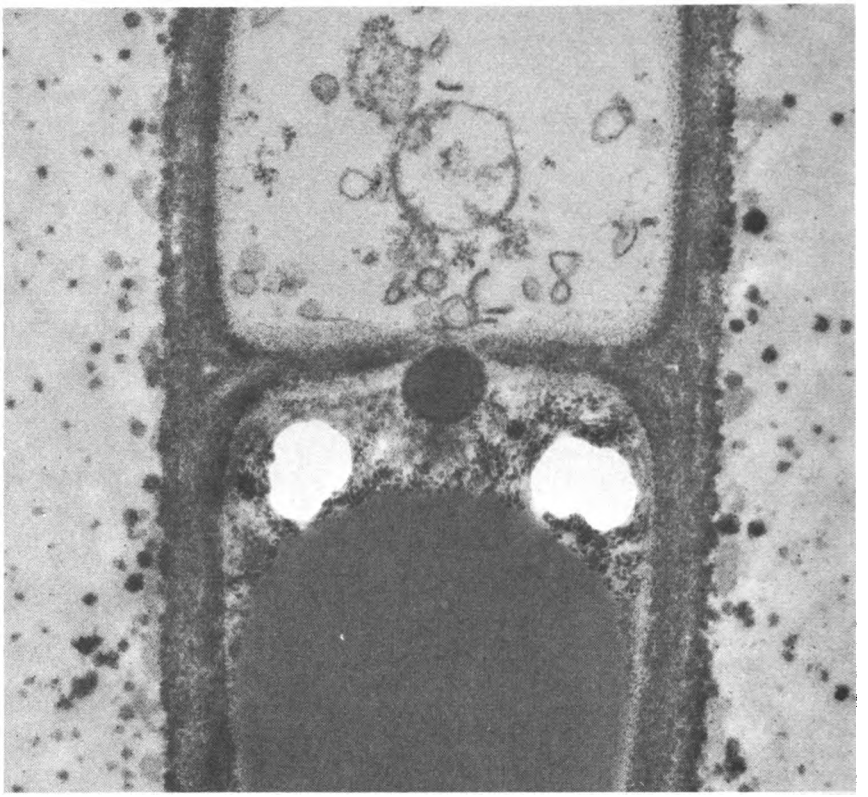

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Figure 3.—Electron photomicrograph of a hyphal septum of *Cenococcum graniforme* (Sow.) Ferd. & Winge. The septal pore has the simple structure characteristic of Ascomycetes (36,000x).

forme have the simple structure typical of Ascomycetes (Moore and McAlear, 1962). As detailed later in this paper, *C. graniforme* is unquestionably related to the genus *Elaphomyces*. It has formed mycorrhizae with numerous tree species in pure culture syntheses, and is known as a mycorrhizal associate of many others (see Trappe 1962, 1964).

Taxa of Mycorrhizal Ascomycetes

Three orders of Ascomycetes include probable mycorrhizal species: (1) Eurotiales: one mycorrhizal genus, *Elaphomyces*, and the imperfect Ascomycete *Cenococcum graniforme;* (2) Tuberales: several families and genera, most species of which are probably mycorrhizal; and (3) Pezizales: only a few of many genera and families proposed as mycorrhizal. A fourth, Helotiales, has been proposed, but evidence is scanty.

Eurotiales

The genus *Elaphomyces* (figs. 1 and 2) is comprised of hypogeous, subglobose, astipitate species, mostly ranging from 1 to 5 cm. in diameter. The asci disintegrate by maturity, leaving the

globose, often darkly pigmented, spiny to reticulate spores as a powdery mass enclosed by the thick, indehiscent peridium. The peridial surface may be smooth or warty, depending on species, but it is initially covered by a "crust" of profuse hyphae. In some species, the crust is readily separable and may not be evident on old specimens or if the ascocarps have not been carefully removed from the soil. In many species, the crust also contains proliferated ectomycorrhizae that may virtually encase the ascocarp.

The mycorrhizal hosts of *Elaphomyces* species are given in the tabulations of the last section of this paper. Ectomycorrhizae are probably formed in all cases. Numerous other papers refer to association between *Elaphomyces* spp. and trees without reference to mycorrhizal association. These have not been included in the list but would extend it substantially. Nonetheless, our knowledge is scanty, considering the wide distribution, substantial number of species, and apparent lack of specificity of these fungi in regard to host.

The taxonomic affinity between *Cenococcum graniforme* and the genus *Elaphomyces* was recognized by Fries and several other early taxonomists (see Ferdinandsen and Winge, 1925). As noted earlier, *C. graniforme* has a typical Ascomycete septal pore (fig. 3). No one seems to have noticed that the anatomy of *Cenococcum* mycorrhiza mantles is replicated, not only on the surface of *Cenococcum* sclerotia, but also on the outer peridial layer of several *Elaphomyces* species (figs. 4–7). In tangential section, these tissues appear as a mosaic of stellate hyphal clusters, a type of formation apparently unknown for any other fungi.

My anatomical studies of type collections of *Elaphomyces* species have revealed this configuration on outer peridia of *E. anthracinus* Vitt., *E. echinatus* Vitt., *E. leucosporus* Vitt., *E. leveillei* Tul., *E. maculatus* Vitt., *E. morettii* Vitt., *E. septatus* Vitt., *E. singaporensis* Corn. & Hawk., and *E. virgatosporus* Holl. All of these species have blackish peridia, and some have crust hyphae identical to those of *C. graniforme*.

Studies are now in progress to determine whether *C. graniforme* is the vegetative mycelium of only one *Elaphomyces* species, a conglomerate of several, or a completely different fungus of *Elaphomyces* origin that has lost its sexual reproductive process. Present evidence points toward conspecificity between *C. graniforme* and *E. anthracinus*. The latter species is the only one of those listed in the preceding paragraph whose distribution would likely approach that of *C. graniforme*. The crust hyphae of *E. anthracinus* are identical to *C. graniforme* hyphae. In several collections of *E. anthracinus* that I have examined, the crust hyphae include typical *Cenococcum* sclerotia, which are occasionally embedded in the peridium and apparently are confluent with the peridial tissue.

Since most of the reported mycorrhizal hosts of *C. graniforme* have already been listed (Trappe, 1964), this fungus is not included in the tabulation at the end of this paper.

Tuberales

The true truffles, order Tuberales (figs. 8–12), are basically differentiated from other Ascomycetes by their persistent asci, which lack a mechanism for forcible spore discharge. Nearly all

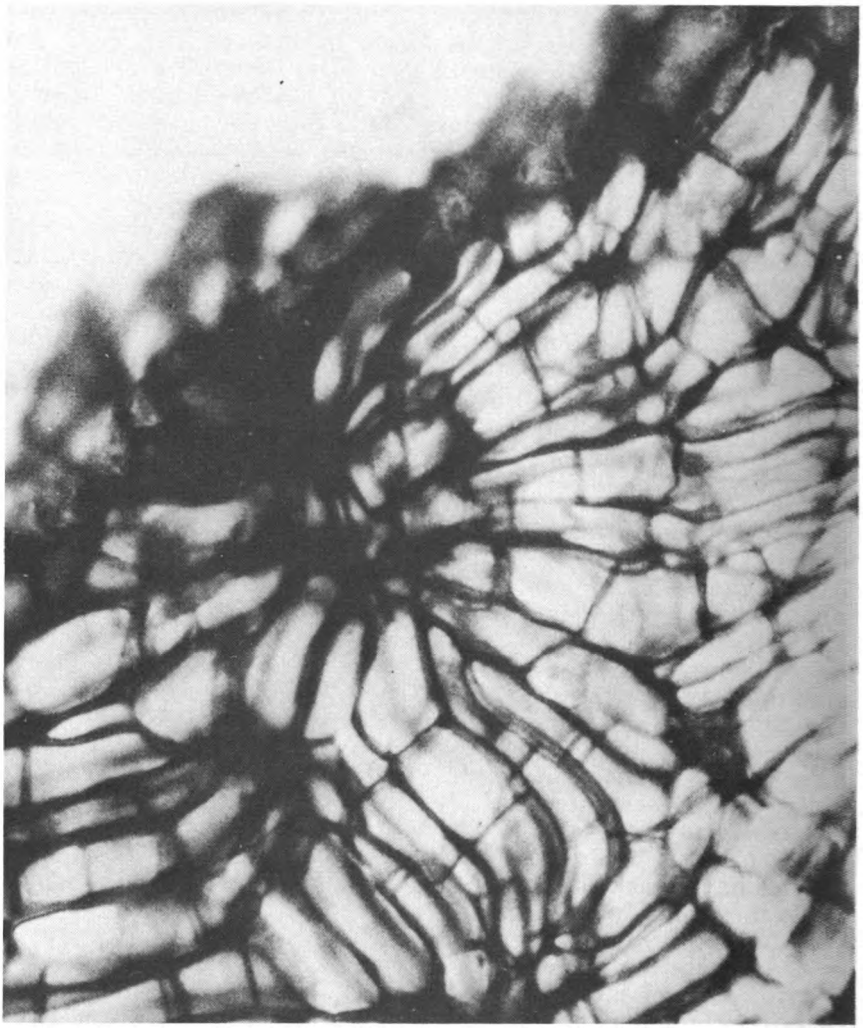

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Figure 4.—Microtomed tangential section of an ectomycorrhizal mantle formed by *Cenococcum graniforme*. The hyphal arrangement is typical (x1200).

are hypogeous and fruit only in association with ectomycorrhizae. Depending on species, they may be hollow, multichambered, veined or solid and may vary in size from a few millimeters to 5 or occasionally more centimeters broad. The ascus-bearing tissue is either completely and persistently enclosed or, at most, exposed by very small openings. Spores vary greatly between species in size, shape, and surface ornamentation.

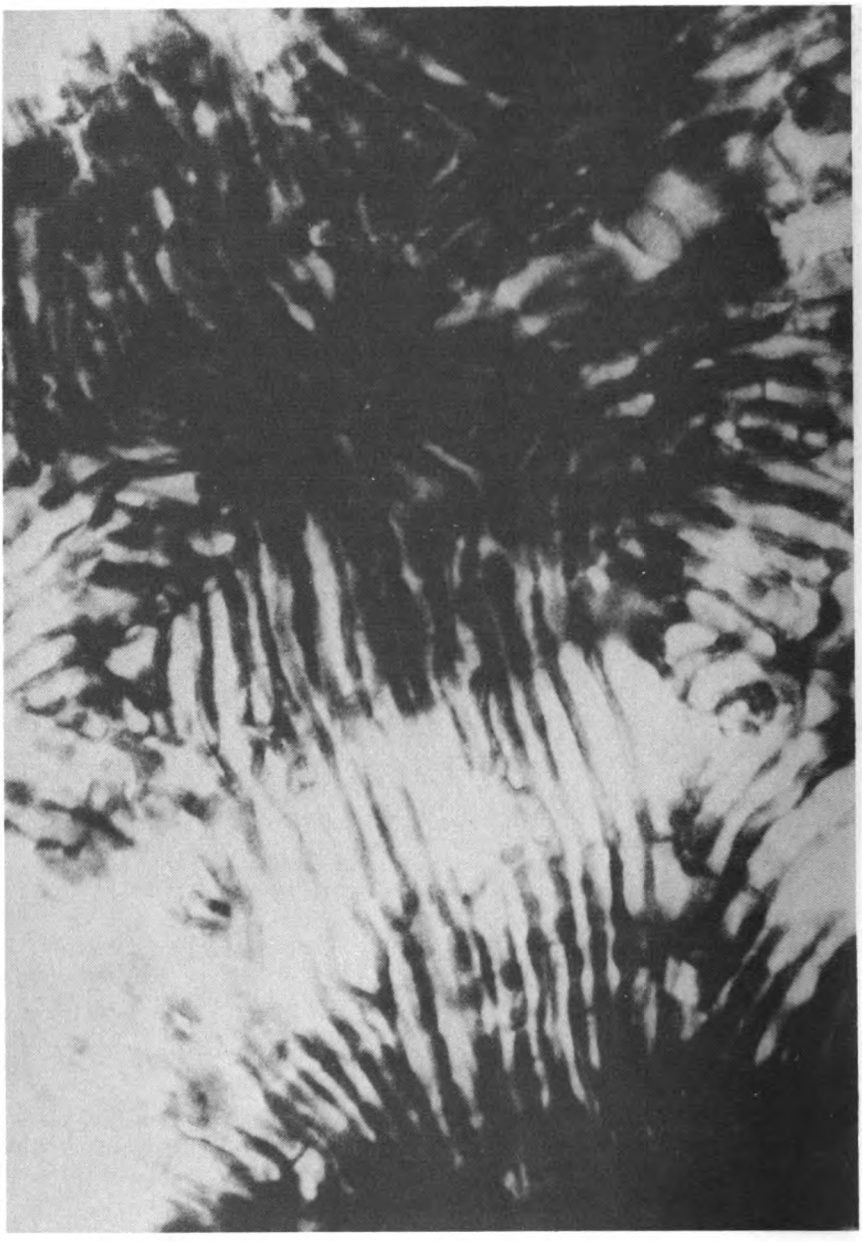
Because of their subterranean habit, Tuberales are seldom encountered by casual collectors. Many species, moreover, are small and drably colored, so are rarely found even by experienced collectors. Several large species, such as *Tuber melanosporum* Vitt. and *Tirmania africana* Chat., are prized as food in Europe and

F-519807

Figure 5.—Microtomed tangential section of the surface of a sclerotium of *Cenococcum graniforme*. The hyphal arrangement resembles that of mycorrhizal mantles formed by this fungus (x1200).

Mediterranean countries of Africa and the Near East. Students of mycorrhizae have good opportunities to find Tuberales in field studies. This can have real value for taxonomic reseach in Asia and the Americas, where relatively little is known about the Tuberales (specimens of hypogeous fungi are hereby solicited from my colleagues in mycorrhiza research—identification will be provided whenever possible.)

Five families are represented in the order Tuberales: Geneaceae, Hydnotryaceae, Balsamiaceae, Tuberaceae, and Terfeziaceae. Most



F-519808

Figure 6.—Hand-cut tangential section from surface of an ascocarp of *Elaphomyces anthracinus* Vitt. The hyphal arrangement resembles that of sclerotia and mycorrhizal mantles formed by *Cenococcum graniforme* (x1200).

species of each family are probable formers of ectomycorrhizae. Many species of Terfeziaceae occur in desert regions in association with roots of members of the Cistaceae. Although a mycorrhizal

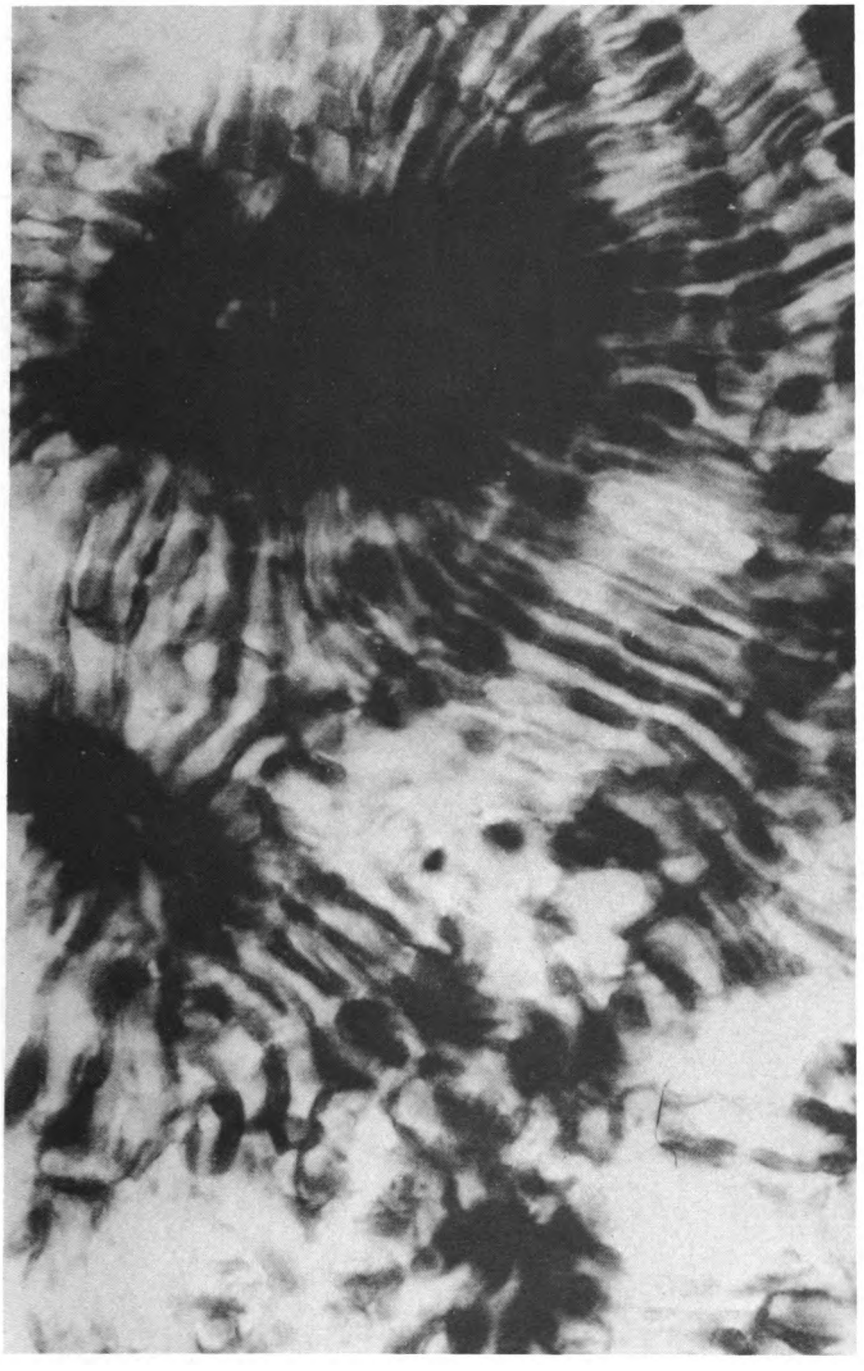


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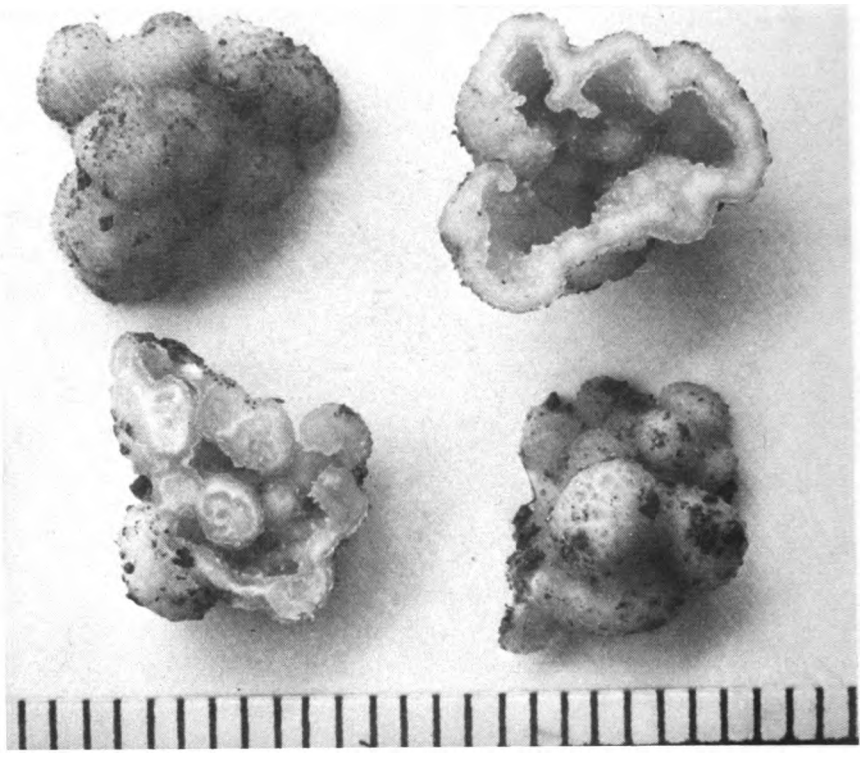
Figure 7.—Hand-cut tangential section from surface of an ascocarp of *Elaphomyces maculatus* Vitt. The hyphal arrangement resembles that of sclerotia and mycorrhizal mantles formed by *Cenococcum graniforme* (x1200).

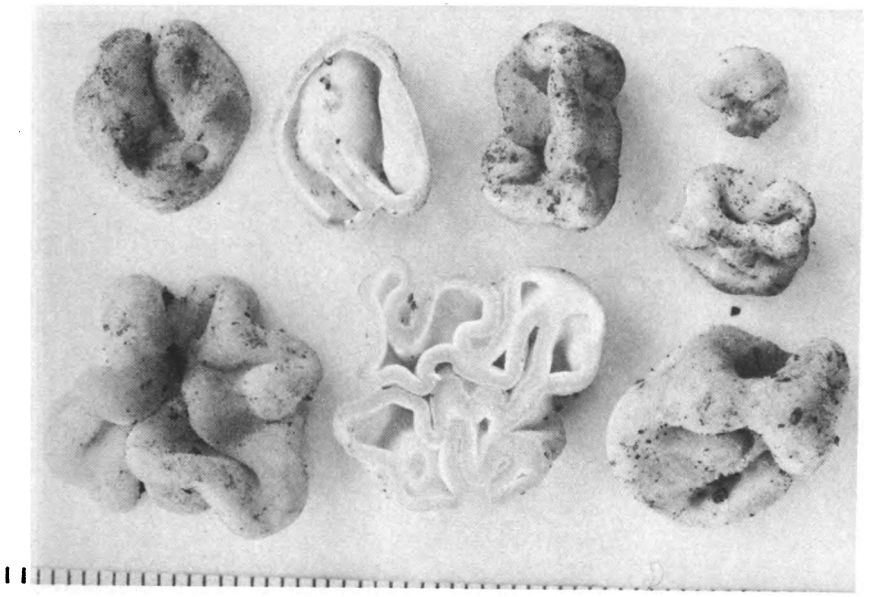


F-519810

Figure 8.—Ascocarps of *Myrmecocystis cerebriformis* Harkn., family Geneaceae, order Tuberales, surface and cross-sectional views. Scale in mm.

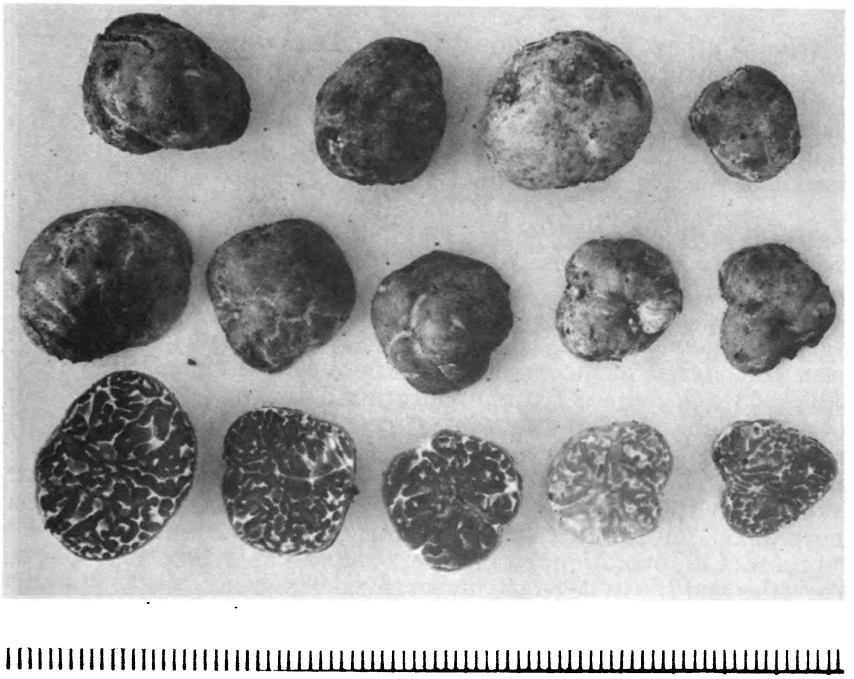
F-519811

Figure 9.—Ascocarps of *Barssia oregonensis* Gilk., family Hydnotryaceae, order Tuberales, surface and cross-sectional views. Scale in mm.



F-519812

Figure 10.—Ascocarps of *Hydnotrya cubispora* (Bess. & Thomps.) Gilk., family Hydnotryaceae, order Tuberales, surface and cross-sectional views. Scale in mm.



F-519813

Figure 11.—Ascocarps of *Tuber californicum* Harkn., family Tuberaceae, order Tuberales, surface and cross-sectional views. Scale in mm.

F-519814

Figure 12.—Ascocarps of *Tuber gibbosum* Harkn., family Tuberaceae, order Tuberales, surface and cross-sectional views. Scale in mm.

relationship has been presumed for the Terfeziaceae, types of mycorrhizae formed have not been studied in detail.

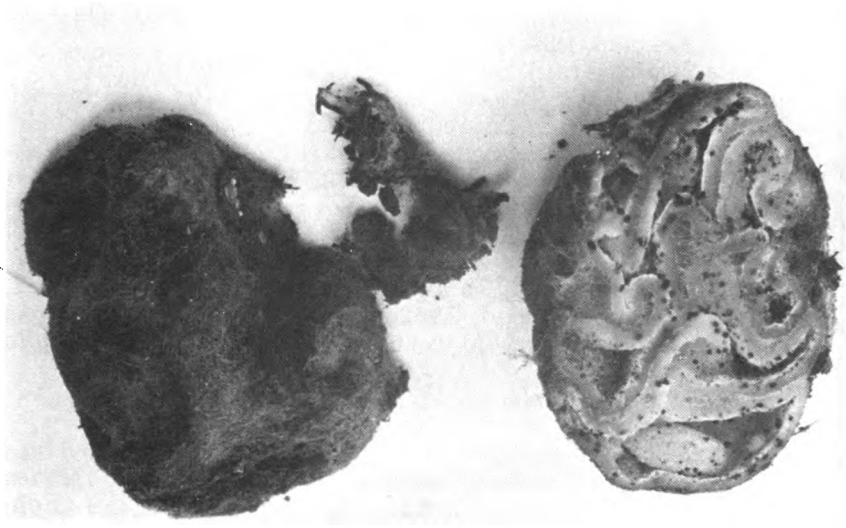
The probable mycorrhizal hosts of Tuberales are listed in the last section of this paper. As in the case of *Elaphomyces*, the list could be extended substantially were it to include associations reported in the literature without specific reference to mycorrhizae.

Helotiales and Pezizales

Relatively few of these fungi have been suggested as mycorrhizal (see list in the last section of this paper). Most appear to be saprophytes or parasites. However, two species in the Pezizales, *Geopora cooperi* Harkn. (fig. 13) and *G. clausa* (Tul.) Burds., have been considered members of the Tuberales until recently (Burdsall, 1968). *G. cooperi* fruits underground among ectomycorrhizae, a typical Tuberales habit, but its spores are forcibly discharged. My observations of this species in the field strongly indicate that it is indeed an obligate ectomycorrhizal fungus. This characteristic, combined with its hypogeous fruiting and a consequently useless mechanism for forcible spore discharge, suggests that *G. cooperi* represents an evolutionary link between the epigeous, saprophytic Pezizales and the hypogeous, mycorrhizal Tuberales.

Summary

Hypogeous Ascomycetes, especially species of *Elaphomyces* and Tuberales, have featured prominently in the literature on mycorrhizae. Experimental evidence for their mycorrhizal habit is



F-519815

Figure 13.—Ascocarp of *Geopora cooperi* Harkn. f. *cooperi*, family Pyronemaceae, order Pezizales, surface and cross-sectional views (black spots on the latter are from a mycoparasite). Scale in mm.

scanty because of the general failure to date of growing them in pure culture. This difficulty in itself is strong evidence that they are not saprophytes. Moreover, they are mostly obligate associates with mycorrhizae, and recent studies have linked the anatomy of specific types of mycorrhizae to similar structures of associated ascocarps. The total evidence now available leaves little room for doubt that Ascomycetes are widely spread mycorrhizal formers.

Cenococcum graniforme, the most intensively studied of the ectomycorrhizal fungi, has been known only in the imperfect stage. By electron microscopy, its hyphae have been shown to possess typical Ascomycete septal pores. It is anatomically analogous to several *Elaphomyces* species and possibly is the vegetative stage of *E. anthracinus*.

Tabulation of Mycorrhizal Associations of Ascomycetes

The mycorrhizal hosts of Ascomycete species are listed below: (1) as reported in the literature, and (2) as additionally indicated by my studies in the Pacific Northwest. In the later case, the ascocarp collections indicated by numbers in parentheses are deposited in the herbarium of Oregon State University.

Order Eurotiales

Family Elaphomycetaceae

Elaphomyces anthracinus Vitt.: *Betula* sp. (Eckblad, 1962), *Fagus sylvatica* (Eckblad, 1962).

E. asperulus Vitt.: *Abies amabilis* (Trappe cols. 955, 1591), *A. procera* (Trappe col. 1713), *Betula* spp. (Eckblad, 1962), *Picea abies* (Eckblad, 1962), *Pinus sylvestris* (Eckblad, 1962), *Pseudotsuga menziesii* (Trappe col. 495), *Quercus* spp. (Eckblad, 1962).

E. cervinus (L. ex Gray) Schlecht.: see *E. granulatus*.

E. granulatus Fr. (syn. *E. cervinus*): *Abies amabilis* (Trappe col. 730), *A. procera* (Trappe col. 492), *Alnus* spp. (Eckblad, 1962), *Betula* spp. (Eckblad, 1962), *Fagus sylvatica* (Eckblad, 1962, Boullard and Dominik, 1966), *Juniperus communis* (Eckblad, 1962), *Picea abies* (Dominik and Pachlewski, 1956; Dominik, 1961; Eckblad, 1962), *Pinus* spp. (Khokhryakov, 1956), *Pinus monticola* (Trappe col. 489), *Pinus sylvestris* (Reess, 1885a and 1885b; Reess and Fisch, 1887; Laing, 1932; Eckblad, 1962; Pachlewski, 1967), *Quercus* spp. (Boullard and Moreau, 1962), *Tsuga heterophylla* (Trappe col. 185).

E. leveillei Tul.: *Corylus cornuta* (Eckblad, 1962).

E. miyabeanus Imai.: *Ulmus japonica* (Imai, 1929).

E. muricatus Fr.: *Abies amabilis* (Trappe cols. 488, 490), *Alnus* spp. (Eckblad, 1962), *Betula* spp. (Eckblad, 1962), *Castanea sativa* (Fontana and Centrella, 1967), *Fagus sylvatica* (Fontana and Centrella, 1967), *Juniperus communis* (Eckblad, 1962), *Picea abies* (Eckblad, 1962), *Pinus sylvestris* (Eckblad, 1962).

E. nopporensis Imai: *Ulmus japonica* (Imai, 1929).

E. reticulatus Vitt.: *Corylus cornuta* (Eckblad, 1962).

E. variegatus Vitt.: *Picea sitchensis* (Trappe col. 592), *Pinus sylvestris* (Reess and Fisch, 1887; Lewton-Brain, 1901), *Tsuga heterophylla* (Trappe col. 567).

Order Tuberales

Family Balsamiaceae

Balsamia nigrens Harkn.: *Pinus jeffreyi* (Trappe col. 1737).

B. platyspora Bk. & Br.: *Tilia* sp. (Bussetti, 1962), *Tilia tomentosa* (Ceruti and Bussetti, 1962).

B. vulgaris Vitt.: *Quercus* spp. (Mattirolo, 1932), *Tilia tomentosa* (Bussetti, 1962).

Family Geneaceae

Genea klotzschii Bk. & Br.: *Carpinus betulus, Quercus petraea, Tilia* spp. (all Fontana and Centrella, 1967).

G. verrucosa Vitt.: *Carpinus betulus* (Fontana and Centrella, 1967).

Myrmecocystis cerebriformis Harkn.: *Pseudotsuga menziesii* (Trappe cols. 504, 517, 694, 714).

Family Hydnotryaceae

Barssia oregonensis Gilk.: *Alnus rubra* (Trappe col. 553), *Pseudotsuga menziesii* (Trappe cols. 512, 514, 515, 696, 713, 764).

Choiromyces meandriformis Vitt.: see *C. venosus*.

C. venosus (Fr.) T. Fr. (syn. *C. meandriformis*): *Betula* spp. (Tichomirow, 1896; Khokryakov, 1956), *Carpinus betulus* (Fontana and Centrella, 1967), *Fagus sylvatica* (Fontana and

Centrella, 1967), *Picea abies* (Tichomirow, 1896), *Pinus* spp. (Khokryakov, 1967), *Pinus sylvestris* (Tichomirow, 1896), *Populus tremula* (Tichomirow, 1896).

Hydnotrya carnea (Corda ex Zobel) Zobel: see *H. tulasnei.*

H. cubispora (Bess. & Thomps.) Gilk.: *Abies lasiocarpa* (Trappe col. 816).

H. tulasnei (Bk.) Bk. & Br. (syn. *H. carnea*) : *Larix decidua* (Peyronel, 1929).

H. variiformis Gilk.: *Abies amabilis* (Trappe col. 852), *A. lasiocarpa* (Trappe cols. 527, 528, 818, 820, 823), *A. procera* (Trappe cols. 524, 724), *Pseudotsuga menziesii* (Trappe col. 523), *Tsuga mertensiana* (Trappe cols. 767, 768, 774, 779).

Family Terfeziaceae

Mukagomyces hiromichii Imai: *Acer palmatum* (Imai, 1940), *Populus deltoides* x *nigra* (McLennan, 1961), *P. nigra* (Imai, 1940).

Picoa carthusiana Tul.: *Pseudotsuga menziesii* (Trappe cols. 526, 1820).

Terfezia claveryi Chat. (syn. *T. transcaucasica*) : *Cistus salvifolius, Helianthus salicifolius* (both Tichomirow, 1896).

T. leonis (Tul.) Tul.: *Helianthemum sessiliflorum* (Rayss, 1959), *Tuberaria vulgaris* (Tichomirow, 1896).

T. transcaucasica Tich.: see *T. claveryi.*

Tirmania africana Chat.: *Helianthemum lippii* (Rayss, 1959).

Family Tuberaceae

Tuber aestivum Vitt.: *Fagus sylvatica* (Frank, 1885; Lubelska, 1953), *Quercus* spp. (Mattirolo, 1933; Khokhryakov, 1956).

T. albidum Pico ex Ceruti: see *T. borchii.*

T. borchii Vitt. (syn. *T. albidum*) : *Corylus avellana* (Fontana and Centrella, 1967), *Fagus sylvatica* (Ceruti, 1965), *Larix decidua* (Ceruti, 1965), *Pinus* spp. (Mattirolo, 1933), *P. pinea* (Ceruti, 1965; Fontana and Centrella, 1967), *P. pinaster* (Ceruti, 1965), *P. strobus* (Scannerini and Palenzona 1967; Ceruti, 1968), *P. sylvestris* (Ceruti, 1965), *Populus* spp. (Ceruti, 1965), *P. deltoides* x *nigra* (Mattirolo, 1934), *Quercus* spp. (Ceruti, 1965), *Quercus petraea* (Fontana and Centrella, 1967).

T. brumale Vitt.: *Quercus* spp. (Khokryakov, 1956), *Tilia* sp. (Fontana and Centrella, 1967.

T. californicum Harkn.: *Pseudotsuga menziesii* (Trappe cols. 503, 691).

T. excavatum Vitt.: *Fagus sylvatica* (Hawker, 1954).

T. ferrugineum Vitt.: *Corylus avellana* (Fontana and Centrella, 1967).

T. gibbosum Harkn.: *Pseudotsuga menziesii* (Trappe cols. 947, 1575, 1588, 1589, 1626, 1627, 1773, 1785).

T. griseum Pers. ex. Fr. (syn. *T. magnatum*): *Abies alba* (Mattirolo, 1910 and 1932; Sella, 1932), *Alnus* spp. (Mattirolo, 1910), *Carpinus betulus* (Mattirolo, 1910), *Castanea sativa* (Mattirolo, 1910), *Cedrus* sp. (Mattirolo, 1910), *Corylus avellana* (Mattirolo, 1909 and 1910; Ceruti, 1965), *Juniperus* spp. (Mattirolo, 1910 and 1932), *Ostrya* spp. (Mattirolo,

1910), *Pinus* spp. (Mattirolo, 1910), *Populus* spp. (Mattirolo 1909, 1932), *P. alba* (Tichomirow, 1896; Mattirolo, 1910; Ceruti, 1968), *P. fastigiata* (Tichomirow, 1896), *P. nigra* (Tichomirow, 1896; Mattirolo, 1910; Ceruti, 1965 and 1968), *P. pyramidalis* (Ceruti, 1968), *P. tremula* (Mattirolo, 1910; 1910; Ceruti, 1968), *Quercus* spp. (Mattirolo 1909, and 1932; Sappa, 1940), *Q. petraea* (Ceruti, 1968), *Q. pubescens* (Ceruti, 1968), *Q. robur* (Ceruti, 1968), *Salix* spp. (Mattirolo, 1909, 1932), *Salix alba* (Mattirolo, 1910; Ceruti, 1965 and 1968), *S. viminalis* (Mattirolo, 1910; Ceruti, 1968).*Tilia* spp. (Ceruti, 1968), *T. europaea* (Ceruti, 1965).

T. levissimum Gilk.: *Pseudotsuga menziesii* (Trappe cols. 529, 532, 690).

T. macrosporum Vitt.: *Corylus avellana* (Fontana and Centrella, 1967).

T. maculatum Vitt.: *Pinus strobus* (Fassi and de Vecchi, 1963; Fassi and Fontana, 1967; Ceruti, 1968).

T. magnatum Pico ex Vitt.: see *T. griseum.*

T. melanosporum Vitt.: *Abies* spp. (Mattirolo, 1910), *Alnus glutinosa* (Ceruti, 1968), *Betula pendula* (Tichomirow, 1896), *Carpinus betulus* (Tichomirow, 1896; Mattirolo, 1910; Ceruti, 1965 and 1968), *Castanea sativa* (Tichomirow, 1896; Mattirolo, 1910; Gaignebet, 1923; Ceruti, 1965), *Cedrus* spp. (Mattirolo, 1910), *Corylus avellana* (Tichomirow, 1896; Mattirolo, 1910; Ceruti, 1965 and 1968), *Fagus sylvatica* (Tichomirow, 1896; Mattirolo, 1910; Ceruti, 1965), *Juglans* spp. (Gaignebet, 1923), *J. regia* (Tichomirow, 1896), *Juniperus* spp. (Gaignebet, 1923), *Picea abies* (Tichomirow, 1896), *Pinus* spp. (Mattirolo, 1910), *P. halepensis* (Tichomirow, 1896), *P. sylvestris* (Tichomirow, 1896), *Populus* spp. (Mattirolo, 1910; Ceruti, 1965 and 1968), *P. tremula* (Tichomirow, 1896), *Quercus* spp. (Gaignebet, 1923; Lubelska, 1953; Ceruti, 1968), *Q. coccifera* (Mattirolo, 1910; Sappa, 1955; Ceruti, 1965), *Q. ilex* (Tichomirow, 1896; Mattirolo, 1910; Peyronel, 1929; Sappa, 1955; Ceruti, 1965), *Q. petraea* (Tichomirow, 1896; Mattirolo, 1910; Sappa, 1955; Ceruti, 1965), *Q. pubscens* (Tichomirow, 1896; Peyronel, 1929; Sappa, 1955; Ceruti, 1965), *Q. robur* (Tichomirow 1896; Mattirolo, 1910; Sappa, 1955; Ceruti, 1965), *Salix* spp. (Mattirolo, 1910; Ceruti, 1965), *Salix alba* (Tichomirow, 1896).

T. murinum Hesse: *Alnus rubra* (Trappe col. 552), *Pseudotsuga menziesii* (Trappe cols. 513, 516, 598, 692, 715, 759, 946, 1819).

T. nitidum Vitt.: *Carpinus betulus, Corylus avellana, Populus* sp. (all Fontana and Centrella, 1967).

T. rufum Poll. ex Fr.: *Corylus* spp., *Quercus* sp. (all Mattirolo, 1935).

Order Helotiales

Family Geoglossaceae

Cudonia circinańs (Pers.) Fr.: *Picea abies* (Khinkova, 1954).

C. confusa Bres.: *Picea abies* (Khinkova, 1954).

Spathularia clavata (Schaeff.) Sacc.: see *S. flavida.*
S. flavida Pers. ex Fr. (syn. *S. clavata*): *Picea abies, Pinus mugo, P. peuce* (all Khinkova, 1954).

Order Pezizales

Family Helvellaceae

Helvella crispa Scop. ex Fr.: *Fagus sylvatica, Quercus* spp. (both Ulbrich, 1936).
H. infula Schaeff. ex Fr.: see *Gyromitra infula* in family Rhizinaceae.

Family Otideaceae

Otidea indivisa Vel. (syn. *Peziza abietina*) : *Picea abies* (Khinkova, 1954).

Family Pyronemaceae

Geopora cooperi Harkn. f. *cooperi*: *Abies grandis* (Trappe col. 123), *Pseudotsuga menziesii* (Trappe cols. 505, 535, 555, 941, 942, 1670).
G. sumneriana (Cooke) comb. ined. (syn. *Lachnea sumneriana, Sepultaria sumneri, S. sumneriana*): *Cedrus* sp. (Heim, 1957), *C. atlantica* (Boullard, 1961), *C. libani* (Guinier, 1949).
Lachnea sumneriana (Cooke) Phil.: see *Geopora sumneriana.*
Sepultaria sumneri (Bk.) Cooke: see *Geopora sumneriana.*
S. sumneriana (Cooke) Mass.: see *Geopora sumneriana.*

Family Rhizinaceae

Gyromitra esculenta (Pers. ex Fr.) Fr.: *Pinus* sp. (Orlos, 1961), *Pinus sylvestris* (Ulbrich, 1936), *Populus tremula* (Ulbrich, 1936).
G. infula (Schaeff. ex Fr.) Quél. (syn. *Helvella infula*): *Picea abies* (Ulbrich, 1936; Khinkova, 1954).

Family Sarcoscyphaceae

Sarcoscypha harzsluszkia Cooke: *Dryas octopetala* (Colla, 1931).
Sarcosoma globosum (Schmid. ex Fr.) Casp. in Rehm.: *Picea abies* (Parmasto, 1958).

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4.

Characterization and Identification of Douglas-Fir Mycorrhizae

B. Zak

The Problem

Despite the many advances in mycotrophy of forest trees during the past 50 years, we still are unable with few exceptions, to differentiate the types of natural ectomycorrhizae. We are unable to define and identify each mycorrhiza as a function of both a known tree species and known fungus. In fact, we are yet unable to simply recognize distinctive mycorrhizae, regardless of fungal partner identities, in the forest and nursery. To both the mycorrhiza worker and to the forester, the kinds of mycorrhizae remain a confusion of colors, sizes, and shapes.

Each year lists of fungi mycorrhizal with various tree species grow longer, including new species, new genera, and even new families and orders (see Trappe, 1962). Unfortunately, most reported associations are based on the weak assumption that consistent sporophore occurrence with a tree species is indicative of a mycorrhizal relationship by the fungus. Some listed associations are based on pure culture syntheses and other more reliable techniques. But where are these mycorrhizae in the forest? How can we recognize them? Except for a few, most are "paper" mycorrhizae, and some may not exist in nature.

A systematic and orderly method of characterizing and classifying forest tree mycorrhizae is badly needed. Each mycorrhiza must be described as fully as possible, with identification of tree and fungus partners. Above all, the mycorrhiza must be given an identity as a biological unit so that we may better explore its varied role in growth and survival of the tree in nature.

Melin's and Dominik's Systems

Melin (1927) first classified ectomycorrhizae, arranging them into four groups based on gross morphology. The plan was originally intended for short roots of pine but later was extended to other tree species. Dominik (1956) followed with a more detailed system, incorporating many more categories and utilizing anatomical features of mycorrhizae, such as mantle design. Neither method, however, has won acceptance. Both have too general and unwieldy groupings and usually ignore not only fungus but even tree species. Both fail to give identity to the mycorrhiza.

Current Work

Recent attempts to characterize and classify ectomycorrhizae are limited to single tree species. Even more important, they define the distinct, individual natural mycorrhiza. Fontana (1962), for ex-

ample, described and illustrated 16 different ectomycorrhizae on roots of 14 species and varieties of *Salix*. Marks (1965) characterized seven mycorrhizae of *Pinus radiata* D. Don, and Rambelli (1967) described and illustrated 10 mycorrhizae on roots of nursery seedlings of the same species. Fungal symbionts, however, were not identified in either study. Chilvers (1968), in a detailed characterization of eight *Eucalyptus* mycorrhizae did include identities of two fungus partners.

Especially significant and noteworthy are studies by Italian workers. They not only describe and illustrate the natural mycorrhiza but also identify the fungus partner. Identification of the latter was based on (1) the proximity and connection of sporocarp to mycorrhiza, (2) comparison of mantle mycelium with mycelium attached to the sporocarp, and (3) comparison of mantle mycelium with mycelium of pure cultures developed from sporophore tissues. Ceruti and Bussetti (1962), for example, identified fungal symbionts of five mycorrhizae of *Tilia* species, and Fassi and de Vecchi (1963) provided names of fungi of four of the five described mycorrhizae of *Pinus strobus* L. nursery seedlings. Fontana and Centrella (1967) named symbiotic fungi of eight mycorrhizae of *Castanea, Carpinus, Fagus, Pinus,* and *Quercus* species. Fungi of four other mycorrhizae were tentatively identified.

Few Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) mycorrhizae have been described in detail. Trappe (1965 and unpublished data) characterized the black mycorrhiza of *Cenococcum graniforme* (Sow.) Ferd. & Winge and a tuberculate mycorrhiza. Trappe (1967) also described four Douglas-fir mycorrhizae synthesized in pure culture by *Hebeloma crustuliniforme* (Bull. ex St. Amans) Quel., *Suillus subolivaceous* Smith and Thiers, *Rhizopogon colossus* A. H. Smith, and *Astraeus pteridis* (Shear) Zeller. They were not, however, related to natural mycorrhizae. Zak (1969 and unpublished data), beginning a series of papers on characterization and classification of Douglas-fir mycorrhizae, definitively described four new mycorrhizae formed by different strains of an unexpected fungal symbiont, *Poria terrestris* (DC. ex Fries) Sacc.

The proposal presented here for the characterization and identification of ectomycorrhizae of Douglas-fir and other Pacific Northwest conifers is based on the natural mycorrhiza. Emphasis is placed on methods for identifying the fungal symbiont and on relatively easy-to-determine characters for use in a key to identify the mycorrhizae. The concepts can be applied to other species.

Characterization of Mycorrhizae

Characterization of natural mycorrhizae should ideally include a complete morphological description with clearly defined illustrations and identities of both tree and fungus partners. If the fungus is unknown but has been isolated in pure culture, a second-best characterization will, instead of the fungus binomial, contain its cultural description and its arbitrary designation. And, if the fungal symbiont is both unknown and not isolated, a third-best characterization is simply an accurate description of the mycorrhiza. This incomplete characterization may be fully useful for many field studies, as pointed out by Chilvers (1968). Actually, it repre-

sents the most important first step of recognizing and describing a distinct mycorrhiza. As additional information is obtained, the characterization can be completed.

Naming Mycorrhizae

Regardless of completeness of fungus identification, each distinct and described mycorrhiza should be named and thereby accorded identity. The following plan is used:

1. Tree binomial + Fungus binomial
2. Tree binomial + Arbitrary designation of isolated fungal symbiont
3. Tree binomial – Mantle color and series number

For example:

1. *Pseudotsuga menziesii* + *Poria terrestris* (blue-staining)
2. *Pseudotsuga menziesii* + Z–14
3. *Pseudotsuga menziesii* – White #1

Identification of Fungal Symbiont

Pure Culture Synthesis of Mycorrhizae

Four methods are available to identify the fungal component of mycorrhizae. First, we may use Melin's (1921) technique, or one of its variations, and synthesize mycorrhizae employing a known fungus isolate obtained from sporophore tissue. Unfortunately, it often is very difficult to produce these structures in an artificial medium and atmosphere even when a fungus is known to be mycorrhizal. With media commonly used, some tree species are more successful in forming mycorrhizae than others. Most pines, for example, produce mycorrhizae in pure culture far more readily than either Douglas-fir or western hemlock (*Tsuga heterophylla* (Raf.) Sarg.). Moreover, the fungi vary much in capacity to enter into mycorrhizal relationship. To illustrate, *Thelephora terrestris* (Ehrh.) Fr. readily forms mycorrhizae with the several species of pine tested and moderately so with Douglas-fir; however, *Lactarius sanguifluus* (Paulet ex) Fr. forms mycorrhizae weakly and with great difficulty with these trees. Use of this method is further limited by our inability to grow a large proportion—probably as much as 40 percent—of mycorrhizal fungi on any known laboratory medium.

But the major disadvantage of pure culture mycorrhiza synthesis is the difficulty, if not impossibility, of relating the mycorrhiza to its natural counterpart in the forest. Very few mycorrhizae, if any, have been positively identified from pure culture specimens. In my experience, a synthesized mycorrhiza, grown in an axenic and artificial environment, significantly differs morphologically from the natural structure more often than not.

Isolation of Fungus

The second method is to isolate the fungal symbiont in pure culture from the mycorrhiza or attached rhizomorphs and compare it with known cultures isolated from sporophore tissues (fig. 1, A-B). It is much better to use isolates from suspect sporophores

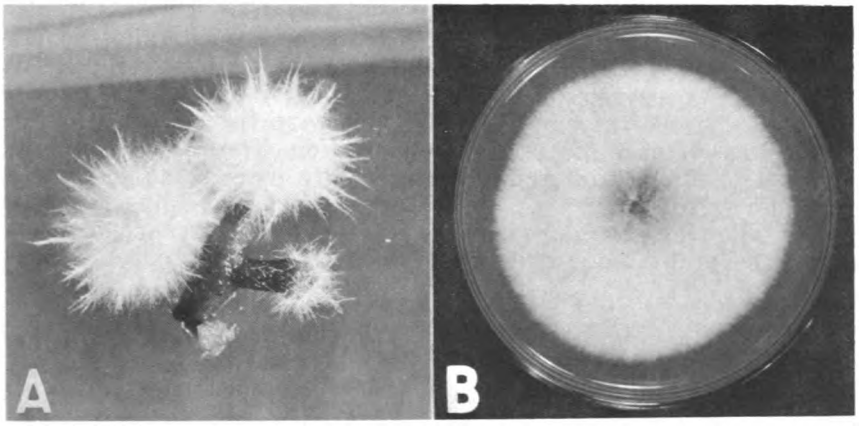

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Figure 1.—Isolation of fungal symbiont from Douglas-fir mycorrhiza. A. Outgrowth of fungus from surface sterilized piece of mycorrhizae on nutrient agar medium. Fungus emerges only from uninjured tissues, especially tips of elements, never from cut surfaces. Wound phenolics probably block growth of fungus from cut ends. 8.7x. B. Petri dish culture of isolated fungus for comparison with known stock cultures. 0.5x.

gathered in the immediate vicinity of the mycorrhiza find than to make comparisons with fungi from a general stock culture collection. The comparison is most definitive if several isolates from several different specimens of the mycorrhiza and sporophores are examined. Use of more than one culture medium can be helpful.

Isolation of the fungal symbiont from the mycorrhiza is not difficult except that, as with the pure culture technique, it is limited to those fungi that grow on available media. When 100 ppm $HgCl_2$ is used for 2–4 minutes (Zak and Marx, 1964) or, better still, 30 percent H_2O_2 for 5–20 seconds (Zak, unpublished data) as surface sterilant, good results are possible. It is important that fresh and initially clean mycorrhizal elements be used and that surface sterilizing be followed immediately by thorough rinsing in sterile water before tissue is plated on nutrient agar medium. During the past 5 years, I have attempted isolations from approximately 25 different Douglas-fir mycorrhizae. Fifteen of these yielded pure cultures of their respective fungal symbionts—all basidiomycetes. Success for individual mycorrhizae ranged from 5 to as high as 100 percent.

Tracing Rhizomorphs and Hyphae to Sporocarp

The third method is to trace rhizomorphs when present or hyphae when possible from mycorrhizae to nearby sporophores. Although tedious and requiring patient probing, the method can, when feasible, provide positive identification of the mycorrhizal fungus. It works best for mycorrhizae of hypogeous fungi whose sporocarps may be found nestled in and connected to a mass of mycorrhizae. For some mycorrhizae, ultraviolet fluorescence may aid tracing of rhizomorphs or hyphae.

Linking Sporocarp to Underlying Mycorrhizae

The fourth and, in my opinion, the easiest and best technique is to compare mycelium at the base of the sporocarp with mycelium

of mycorrhizae directly underlying the sporophore. Cursory application of the method has been used in the past to compile listings of possible tree-fungus mycorrhizal associations. However, careful and judicious comparison of several distinctive macro- and microscopic features of mycorrhizal and sporocarp tissues can provide conclusive evidence of fungus identification. The mycorrhizae in figure 2, *A-D* were thus identified recently in Douglas-fir forests in western Oregon.

The following procedure is used. First, a fresh sporocarp or, preferably, several closely growing sporocarps are selected and care-

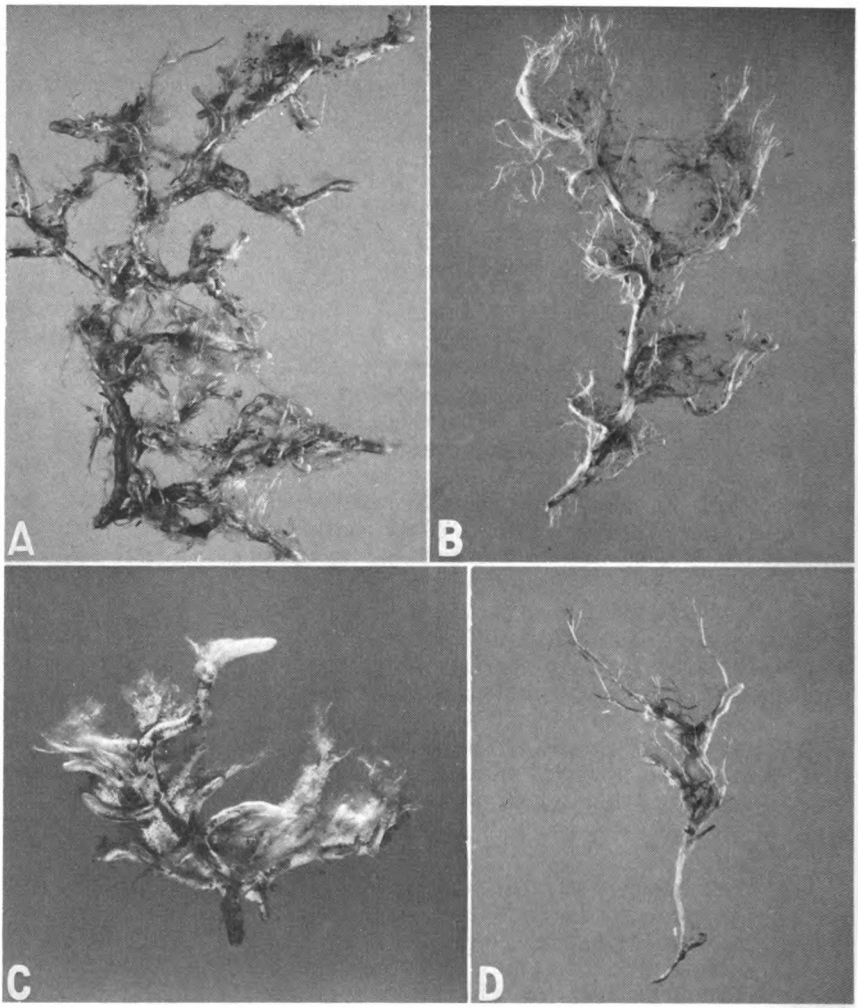

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Figure 2.—Douglas-fir and madrone mycorrhizae whose fungal symbionts were identified by linking sporocarps to underlying mycorrhizae. A. *Pseudotsuga menziesii* + *Cortinarius croceifolius* Peck; B. *P. menziesii* + *C. semisanguineus.* (Fr.) Gill.; C. *P. menziesii* + *C. sanguineus* (Wolf. ex Fr.) Fr.; D. *Arbutus menziesii* Pursh (madrone) + *C. croceifolius.* 3.5X.

fully lifted to retain attached mycelium. The latter is briefly examined with an X10 hand lens for color and gross character. Next, the soil within a circumference of about 6 inches of the sporocarps and to a depth of at least 6 inches is searched for mycorrhizae with similar attached mycelium or, at least, with a mantle of similar coloring. Sporocarp and mycorrhiza samples are then brought to the laboratory and subjected to careful examination. The following features are compared:

1. *Absence or presence of rhizomorphs and, if present, their macroscopic and microscopic character.* Rhizomorphs are usually quite distinctive and when present provide excellent evidence to establish the mycorrhiza-sporocarp association
2. *Macroscopic and microscopic character of mycelium attached to mycorrhizae.* Most Douglas-fir mycorrhizae have some surrounding mycelium; but if none is present and mycorrhiza elements are smooth as, for example, those of *Pseudotsuga menziesii* + *Lactarius sanguifluus* (fig. 3, A-B), then at least color of sporocarp mycelium may be compared to that of the mantle.
3. *Chemical reagent color reaction test of fungus tissues.* Reagents such as those listed by Singer (1962) are applied to the mycorrhiza and to the basal tissue of the suspect sporocarp on a white, glazed, porcelain plate; color reactions that develop within 5 min. are noted. Table 1 summarizes tests with fungal tissues of Douglas-fir mycorrhizae (fig. 4, A-D) and respective *Poria terrestris* fungal symbiont sporo-

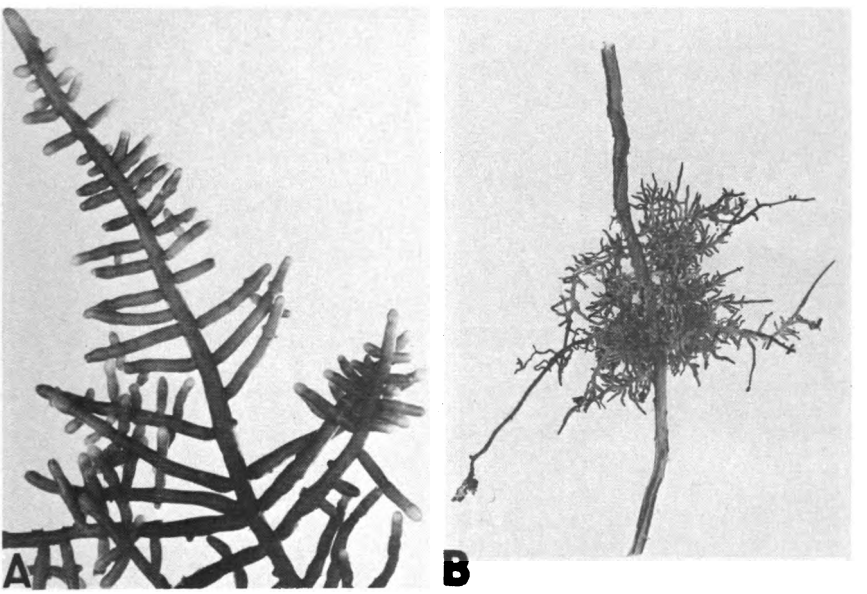

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Figure 3.—Douglas-fir mycorrhizae, *Pseudotsuga menziesii* + *Lactarius sanguifluus*. First dull orange, the smooth elements gradually become deep verdigris. A. Closeup of compound pinnate fan. 3.5X. B. Cluster of mycorrhizae along root. 0.70X.

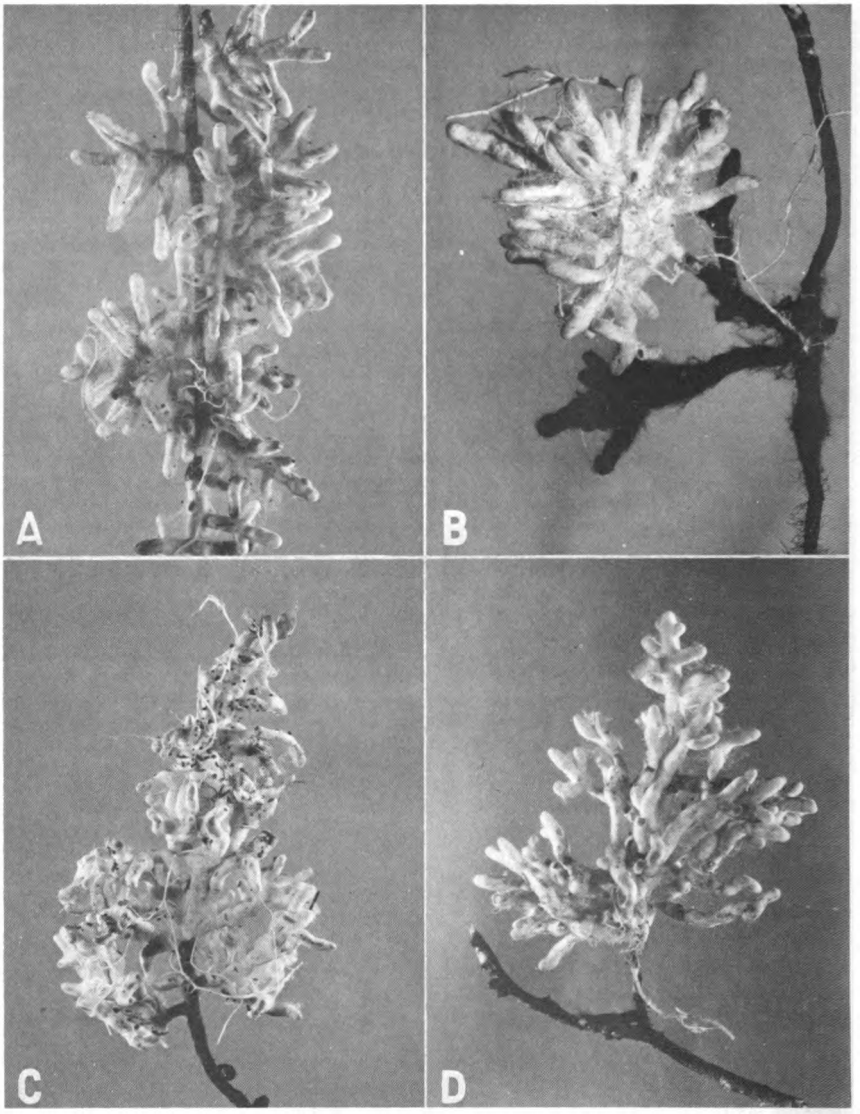

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Figure 4.—Four distinct Douglas-fir mycorrhizae, each formed by a different strain of *Poria terrestris*. A. *Pseudotsuga menziesii* + *P. terrestris* (blue-staining). B. *P. menziesii* + *P. terrestris (orange-staining)*. C. *P. menziesii* + *P. terrestris (rose-staining)*. D. *P. mensiesii* + *P. terrestris* (yellow). 3.5X.

carps. Table 2 details reactions for rhizomorphs and mycelia attached to mycorrhizae of Douglas-fir and madrone and those attached to sporocarps of respective *Cortinarius* sp. symbionts. Although color reaction of the mycorrhiza mantle is usually characteristic and distinctive, it may differ

somewhat from that of rhizomorphs and mycelium because of interfering reactions by underlying root tissue.

Fungal tissues of many Douglas-fir mycorrhizae exhibit positive color reactions with the few reagents now in use. Those of some mycorrhizae react to only one reagent, others to several. Obviously, the more reagents which react with fungal tissues of a specific mycorrhiza, the greater the significance of the mycorrhiza-sporophore association. Hence, a search for more reagents should be highly rewarding. An especially good source for discovering new ones is Feigl's (1956) treatise on spot tests in organic analysis.

4. *Fluorescence of fungi.* Tissues of most fungi of ectomycorrhizae fluoresce in long-wave ultraviolet light in varying colors and intensities. A comparison of fluorescence of the mycorrhizae with basal tissues of the overlying sporophore can significantly aid fungal symbiont identification. For exaample, the mantle and surrounding mycelium and rhizomorphs of *Pseudotsuga menziesii* + *Cortinarius semisanguineus* fluoresce a brilliant golden-yellow, (fig. 5, A-B), identical in color and intensity to that of basal tissues of the fungal symbiont sporophore.

An especially striking and unusual character may alone suffice to establish identity of the mycorrhizal fungus. An example is a Douglas-fir mycorrhiza formed with *Inocybe xanthomelas* Boursier & Kühner. Both the base of the sporophore and the regularly pinnate, white mycorrhiza are enveloped by identical, white, fleecy mycelium among which are interspersed numerous brightly white sclerotia-like bodies resembling tiny pearls (fig. 6, A-B). Most Douglas-fir mycorrhizae, however, will require four or more clearly defined characters to reliably establish the mycorrhiza-sporophore association. As a rule of thumb, these should agree in at least three separate mycorrhiza-sporophore collections.

Aside from the relative simplicity and freedom from difficult and time-consuming tree and fungus culturing, the principal advantages of this method are: (1) the natural, rather than the synthe-

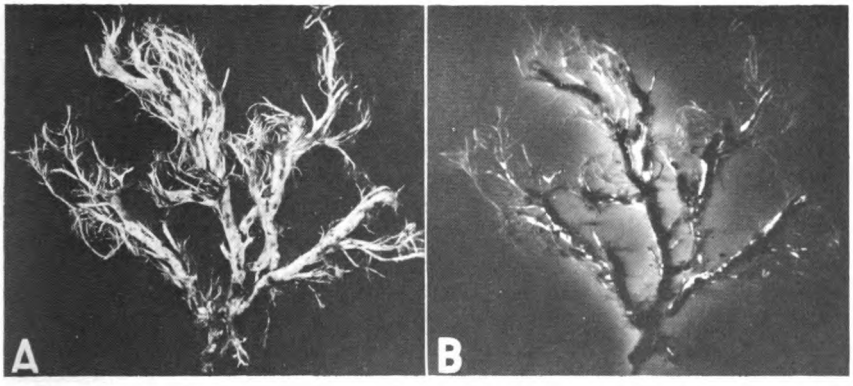

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Figure 5.—Long-wave ultraviolet light fluorescence of *Pseudotsuga menziesii* + *Cortinarius semisanguineus* mycorrhiza. A. In normal light. B. In 3660A ultraviolet light. Fluorescence is bright golden-yellow. 3.5X.

Table 1.—*Summary of color reactions of fungal tissues of* Pseudotsuga menziesii + Poria terrestris *mycorrhizae and respective fungal sporocarps to chemical reagents*

Chemical reagent [1]	Mycorrhiza—according to *P. terrestris* strain [2]			
	Blue-staining	Orange-staining	Rose-staining	Yellow
Chlorovanillin	Purple	—	Pink-red	—
$FeSO_4$	—	—	Blue-grey	—
H_2SO_4, conc.	Purple	—	—	—
KOH, 15%	Pink	Brown	Maroon	Orange
Melzer	Green	—	—	—
NH_4OH, conc.	—	Brown	—	Orange
Sulfoformol	Pink	—	Yellow	—

[1] According to Singer (1962), p. 82–94.

[2] Each strain is distinguished by color of sporocarp.

Table 2.—*Color reactions of rhizomorphs and mycelia attached to Douglas-fir* (Pseudotsuga menziesii) *and and madrone* (Arbutus menziesii) *mycorrhizae and to sporocarps of respective symbiotic fungi to chemical reagents*

Chemical reagent [1]	Mycorrhiza: tree + fungal symbiont			
	Pseudotsuga menziesii + *Cortinarius croceifolius*	*P. menziesii* + *C. sanguineus*	*P. menziesii* + *C. semisanguineus*	*Arbutus menziesii* + *C. croceifolius*
$FeSO_4$	Bruise points and sharp bends along rhizomorphs turn green in 3–5 min., deeper green in 10 min.	—	—	Same as for *P. menziesii* + *C. croceifolius*
Guaicol	—	Golden yellow-orange pigment immediately begins to seep away from rhizomorph and mycelial tissues into surrounding liquid.	—	do.
KOH, 15%	Rhizomorphs and mycelium turn pinkish-brown to reddish-orange in 1 min.	Rhizomorphs and mycelium immediately turn an orange-red; pigment of same color seeps into surrounding liquid.	Rhizomorphs and mycelium immediately turn a deep purple.	do.
NH_4OH, conc.	Similar to 15% KOH.	Same as for 15% KOH but color is more a deep wine-red.	Rhizomorphs and mycelium immediatetly turn a deep reddish-purple.	do.

[1] According to Singer (1962), p. 82–94.

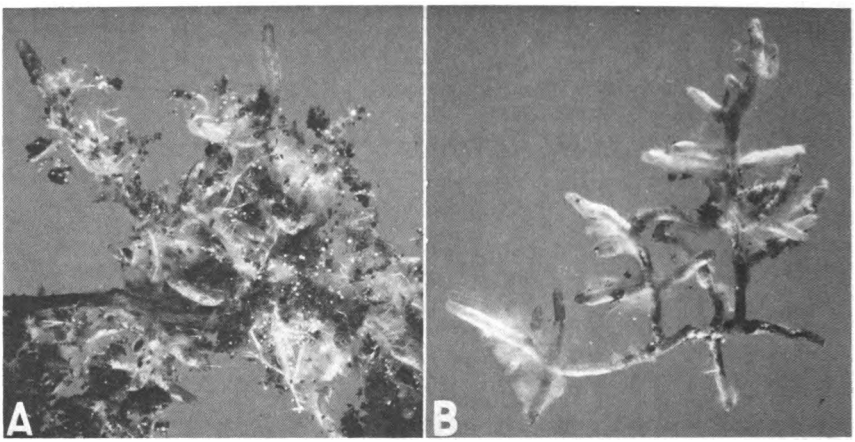

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Figure 6.—Douglas-fir mycorrhiza, *Pseudotsuga menziesii* + *Inocybe xanthomelas*. A. As removed from soil. Note numerous white, sclerotia-like bodies interspersed among surrounding mycelium. B. Removal of soil and most of surrounding mycelium reveals pinnate structures. 3.5X.

sized, mycorrhiza is identified; and, (2) identification of the fungus symbiont is based on the sporophore rather than on difficult comparison of vegetative cultures.

Its main disadvantage is that it cannot be used always and everywhere but is dependent on the presence of sporophores. It is best used in forests that have regular and abundant sporophore production such as those of the Pacific Northwest. Even in these areas some mycorrhizal fungi may pose problems by fruiting erratically and infrequently.

Key for Identification of Mycorrhizae

Characters to be used in a key for identification of mycorrhizae should be simple and easy to determine but fairly stable. If the key is to be practical and meaningful, it should rely on simple preparations and on simple tools. Characters which may be useful in such a key are here ranked by importance according to their ease of determination and stability:

1. *Color of mantle*—This most obvious character which first strikes the eye is quite stable—as much as most biological materials—if we recognize that change may occur with age. Some mycorrhizae, as those formed with *Cenococcum graniforme*, remain the same color throughout their lifetime. Others are first one color, changing gradually with age to another. An example is *Pseudotsuga menziesii* + *Lactarius sanguifluus*. Dull orange when young, this mycorrhiza later becomes a deep verdigris. Others, such as *P. menziesii* + *Poria terrestris* (blue-staining), are one color when young but acquire discrete stains as they become older. Uusually, one can observe all developmental colors in a single collection unless the mycorrhizae are the first or last of the season.

2. *Form*—Douglas-fir mycorrhizae are digitiform or ramiform to weakly or strongly, pyramidally pinnate. Further, pinnate fans may occur singly or be loosely or tightly clustered, and, in one mycorrhiza, they are firmly packed into tubercles.
3. *Surrounding mycelium*—This may be absent or present and includes density and gross character.
4. *Attached rhizomorphs*—They may be absent or present. Note also their abundance and gross character. Color usually is the same as the mantle and surronding mycelium. Form of rhizomorphs is quite variable and characteristic. Some are flat, even sheetlike, and feathery or wefty; others are round or oval in cross-section and threadlike. Surface may be smooth, fibrous, cottony or granular. Rhizomorphs of most mycorrhizae are free and connect to the mantle by attached mycelium, at least when the mycorrhiza is young; others attach directly to the mantle and lengths of some appear almost glued to the mantle.
5. *Individual elements*—Form is straight to tortuous. Average diameter varies little among Douglas-fir mycorrhizae, but evenness of diameter may be characteristic.
6. *Texture of mantle*—Texture may be smooth, felty, velvety, cottony, warty, pitted, or granular.
7. *Chemical reagent color reaction*—Reagents are applied to the clean mycorrhiza as previously described, and color change which occurs within 5 minutes is noted. Some reactions are immediate, others require several minutes. Mantle reaction may be quite characteristic but may vary somewhat with age of the mycorrhiza.
8. *Fluorescence*—The mantle and, if present, the surrounding mycelium and rhizomorphs of most Douglas-fir mycorrhizae fluoresce under long-wave ultraviolet light in varying intensities and colors. It is a stable and useful character, but it may vary somewhat with age of mycorrhiza.
9. *Habitat*—Some Douglas-fir mycorrhizae, such as *Pseudotsuga menziesii* + *Cenococcum graniforme,* can be found in both mineral soil and decayed wood and litter. Others may occur in one or the other medium. In my study (Zak, 1969 and unpublished data) of distinct mycorrhizae formed by four different strains of *Poria terrestris, P. menziesii* + *P. terrestris* (blue-staining) was found mostly in decayed wood and only occasionally in mineral soil. *P. menziesii* + *P. terrestris* (orange-staining) and *P. menziesii* + *P. terrestris* (rose-staining) were found in decayed wood only; and *P. menziesii* + *P. terrestris* (yellow), in mineral soil immediately adjacent to decayed wood containing sporocarps of *P. terrestris* (yellow). My collections of *P. menziesii* + *Cortinarius croceifolius, P. menziesii* + *C. sangineus,* and *P. menziesii* + *C. semisanguineus* have come largely from decayed wood buried in soil.
10. *Odor, taste*—Very few Douglas-fir mycorrhizae have a noticeable odor or taste and then they are only vaguely mushroomlike. An exception is an olive, irregularly pinnate mycorrhiza formed by an as yet unknown fungus (Zak, 1967). It emits

a strong, acrid odor. In fact, presence of the mycorrhiza in a mass of soil and roots is readily detected by this smell.

Microscopic examination, when necessary, should be limited to simple preparations. A very useful character, as Chilvers (1968) has pointed out, is the anatomy of rhizomorphs when present, especially character of surface hyphae. When clearly definable and distinctive, microscopic features of the surrounding mycelium may be helpful; these include: presence or absence and size and shape of clamp connections, diameter of hyphae, their wall thickness, and presence of incrustations and whether these dissolve away in KOH or other solutions. Mantle and, to some degree, Hartig net designs may be distinctive, but, to see these clearly, more elaborate preparations are usually necessary and examination is time consuming.

Use of the foregoing "key" characters to define ectomycorrhizae is illustrated in Table 3 by the four distinct Douglas-fir mycorrhizae formed with different strains of *Poria terrestris*. The orange-staining strain has also been found mycorrhizal with *Tsuga heterophylla* (fig. 7).

Discussion

In the past, largely because of the difficulty of viewing forest tree mycorrhizae in their natural soil environment, they have generally been regarded as rather confusing and nondescript root structures lacking sufficient distinction to receive identities. But as we become more familiar with the natural mycorrhiza, we begin to see many clear morphological differences which can be used to accurately separate and identify various forms. In fact, even fungi which have similar sporocarps may, because of physiological differences, produce quite distinct mycorrhizae with roots of the some tree species. The four *Poria terrestris* strains which form a different Douglas-fir mycorrhizae previously discussed, are an example.

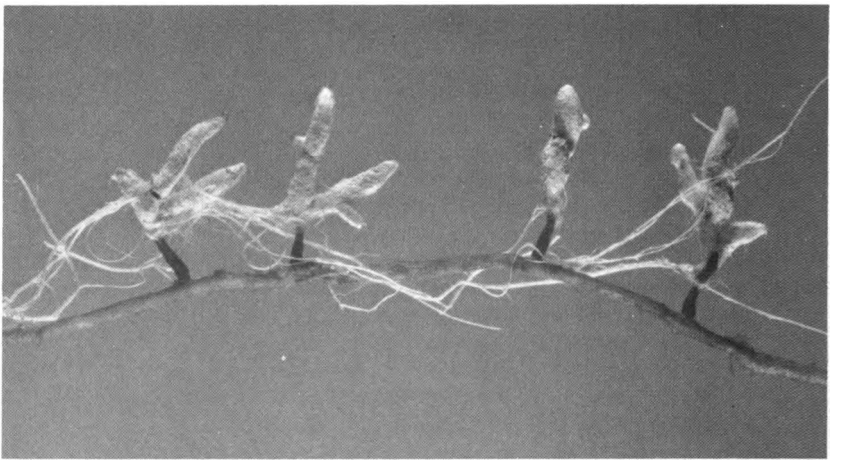

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Figure 7.—Western hemlock mycorrhiza, *Tsuga heterophylla* + *Poria terrestris* (orange-staining), found in decayed wood in western Oregon. 5X.

Table 3.—*Summarized characterization of four distinct, Douglas-fir ectomycorrhizae, each formed with a different strain of* Poria terrestris

Character	Mycorrhiza—according to *P. terrestris* strain [1]			
	Blue-staining	Orange-staining	Rose-staining	Yellow
Color of mantle (X10)	White, later acquiring blue-green, blue and purple stains	White, later acquiring orange stains	White	Yellow with green tint
Form (X10)	Pinnate, single, and clustered	Pinnate, single, and clustered	Pinnate, single, and clustered	Pinnate, single, and clustered
Surrounding mycelium (X10)	Abundant; white; delicate, gossamery	Abundant, white, coarse	Moderately abundant, white, fine	Little to abundant, yellow with green tint, coarse, resembles algal filaments
Rhizomorphs (X10 and X400)	Abundant, white with blue-purple staining, characteristic surface "lacey" hyphae	Abundant, white, then dull yellow	Abundant, white, characteristic stag-horn surface hyphae	Moderately abundant, dull yellow with orange stains
Elements (X10)	Straight to mildly tortuous, diameter regular	Straight, diameter regular	Tortuous, diameter regular	Straight to tortuous, diameter regular
Texture of mantle (X10)	Finely granular, almost powdery	Coarsely felty	Finely granular	Finely crusty
Color reaction to chemicals—number reactions [2] (X10)	5	2	4	2
Fluorescence of mantle, 3660 A UV (X5)	Bright white	Bright white	Dull pinkish-white to dull rose	Dull yellow-olive, 1–2 mm ends of elements a dull orange
Habitat	Decayed Douglas-fir wood and mineral soil	Decayed Douglas-fir wood only	Decayed Douglas-fir wood only	Mineral soil adjacent to decayed Douglas-fir wood
Odor, taste	None	None	None	None

[1] Each strain is distinguished by color of sporocarp.
[2] According to Singer (1962), p. 82–94; see table 1.

Some mycorrhizae have, in my opinion, a more stable morphology than sporocarps of their respective fungal symbionts. An example is *Pseudotsuga menziesii* + *Cortinarius croceifolius*. Although mycorrhizae of seven different samples collected in western Oregon exhibited no discernible differences in color or structure, the overlying fungal symbiont sporocarps displayed moderate variation in color, size, and shape. A possible explanation is that the mycorrhiza is exposed to a less variable environment than the sporocarp. Possibly the distinctiveness and stability of underlying mycorrhizae may aid taxonomists in identifying sporocarps of mycorrhizal fungi.

Some have suggested that site variation may complicate and confuse characterization and establishment of identities of mycorrhizae by inducing much morphological change in the same tree-fungus structure. Those that I have observed in widely divergent habitats in western Oregon, however, display no morphological differences. For example, several Douglas-fir mycorrhizae, one of which is *Pseudotsuga menziesii* + *Cenococcum graniforme*, are formed both in mineral soil (pH 5.5) and in brown-rotted Douglas-fir wood (pH 3.8). In each case, the soil mycorrhiza is identical to its decayed wood counterpart. We may, however, encounter variation in a mycorrhiza when the fungal symbiont is highly variable and produces several physiologically different strains.

Summary

Mycorrhizal research is sorely hampered today by the lack of a practical system to characterize and identify natural mycorrhizae of forest trees. Ideally, identification of mycorrhizae should include both tree and fungus species. However, not only are we unable to name most fungal components, we are yet unable to even distinguish and recognize the distinct mycorrhizae.

A proposal for characterizing and identifying ectomycorrhizae of Douglas-fir and other Pacific Northwest conifers is discussed. Of several methods available to identify the fungal symbiont, that of linking the sporocarp to underlying mycorrhizae by rigidly comparing respective mycelia is deemed best and easiest. An important advantage is that this method is applicable to mycorrhizae whose fungi cannot yet be grown on laboratory media. Its use, however, is limited by sporocarp production.

Simple-to-determine yet relatively stable feaures of ectotrophic mycorrhizae are examined for possible use in an identification key. These include (1) color of mantle, (2) form, (3) presence or absence and character of surrounding mycelium, (4) presence or absence and character of attached rhizomorphs, (5) character of individual elements, (6) mantle texture, (7) chemical reagent color reaction, (8) fluorescence in long-wave ultraviolet light, (9) habitat, and (10) odor and taste. Beginning with tree species, the proposed key will lead the user to mycorrhiza identification which may include identity of the fungal symbiont.

Acknowledgment

Appreciation is expressed to Dr. J. M. Trappe, Pacific Northwest Forest and Range Experiment Station, Forest Service, U.S. Depart-

ment of Agriculture; Prof. J. L. Lowe, State University of New York, Prof. R. L. Gilbertson, University of Arizona; and Prof. D. E. Stuntz, University of Washington, for identification of sporophores of many of the fungi discussed.

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5.

Morphology of Ectendomycorrhizae in *Pinus resinosa*

Hugh E. Wilcox

The distinctions between ectomycorrhizal and ectendomycorrhizal infection in pine are still poorly understood. Mikola (1965) clarified the occurrence of these two types by pointing out that ectendomycorrhizae in pine are almost exclusively confined to 1- to 3-year-old seedlings in nursery soils. Pine ectomycorrhizae, in contrast, he found mostly in plantations and natural forest stands. He studied the change in mycorrhizal structure following transplantation and noted that usually the ectendomycorrhizal fungus did not enter the new growth which was immediately infected by a fungus producing an ectotrophic net. Mikola concluded that unknown factors in forest soils favor the indigenous fungi and inhibit the ectenomycorrhizal species. This weak competitive ability in forest soils is puzzling in view of the wide ecological amplitude of the ectendomycorrhizal fungus in regard to light intensity and soil fertility, acidity and humus content.

Although it appears most likely that the two types of mycorrhizal infection represent a competition between different fungi, it is still possible that the same fungal species may produce an ectendomycorrhizal infection at one time and an ectomycorrhizal at another, as also suggested by Mikola (1965). It appeared to me, from investigations of red pine (*Pinus resinosa* Ait.), that this latter possibility had some merit. In the report of these investigations (Wilcox, 1968), I pointed out that first-order laterals of 2–0 red pine seedlings that have broken dormancy often show intercellular and intracellular infection in the older root portion and only intercellular infection in the new increment. Thus, the intercellular infection always preceded the intracellular. Also in pine seedlings ectomycorrhizae sometimes arose from long-roots with a heavy ectendomycorrhizal infection. These ectomycorrhizae appeared to be considerably smaller in diameter than those which were ectendomycorrhizal. Although appearances favored the existence of different fungal symbionts, the possibility existed that the ectendomycorrhizal fungus also occupied these smaller short-roots, and the intracellular infection was either delayed or did not form under the conditions existing in these roots.

In view of the above uncertainties, a more thorough examination of ectendomycorrhizae was made and comparisons were made to unequivocal ectotrophic types. These investigations have resulted in a better understanding of the morphology and development of ectendomycorrhizae in nursery seedlings. I would like to summarize some of these observations for you.

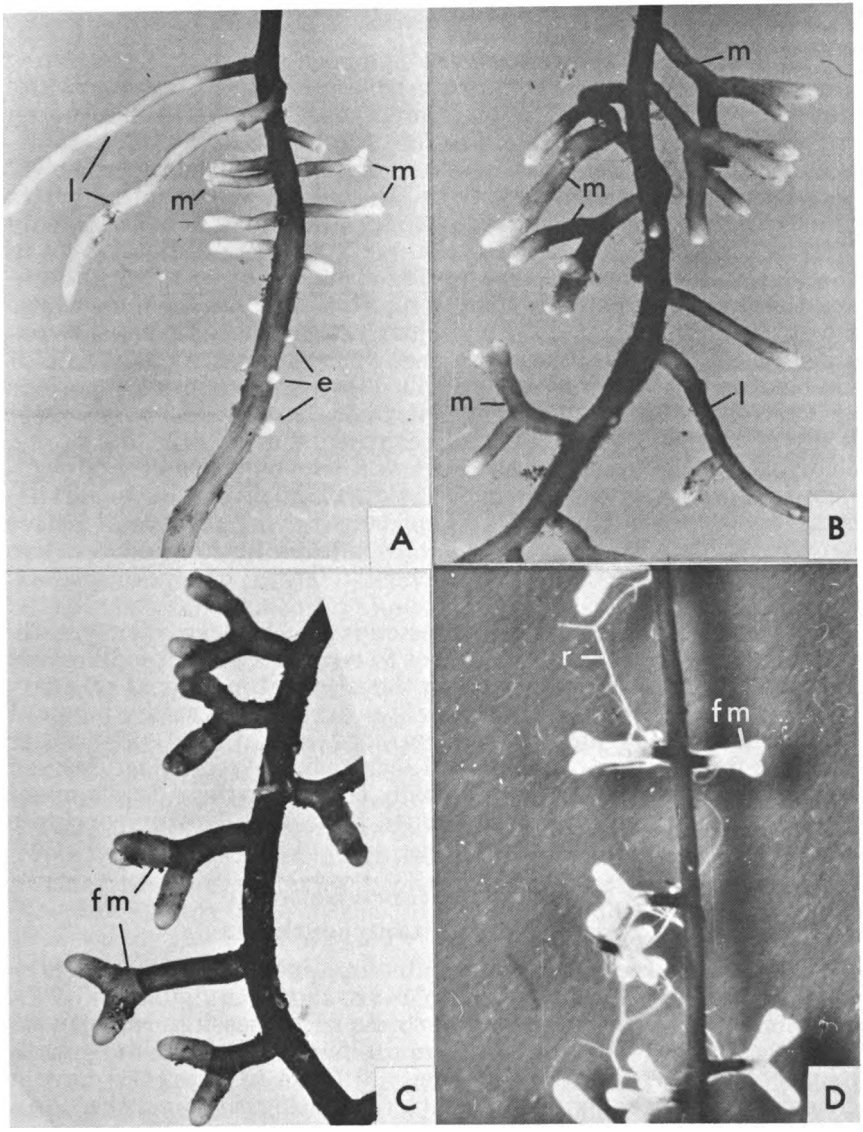

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Figure 1.—Comparison of ectendomycorrhizae and ectomycorrhizae in red pine: *e*, emerging lateral roots; *fm*, fungal mantle; *m*, mycorrhizae; *l*, long-root branches; *r*, rhizomorphs.—A. Initial development of ectendomycorrhizae in 2-year-old seedling, 5.2X.—B. Ectendomycorrhizae at an intermediate stage of development. Growth is still active and no fungus mantle is evident, 6.5X.—C. Ectomycorrhizae with thick, compact pseudoparenchymatous mantle. No conspicuous hyphal projections are evident, 11.X.—D. Ectomycorrhizae with conspicuous white mantle and connecting rhizomorphs, 4.5X.

External Appearance of Ectendomycorrhizae

Typical ectendomycorrhizae are shown in figure 1, A & B. They are generally smooth, without a visible mantle and appear white for the first 2–3 mm after their emergence. They gradually turn amber and retain this color for the first 2–3 months of their development. Subsequently they either uniformly darken, become inactive, and are lost by attrition, or they undergo alternating cycles of activity and dormancy, gradually forming clusters of repeatedly bifurcate mycorrhizae. Figure 1B shows an intermediate stage in the development of these clusters. The cyclic behavior sometimes results in mycorrhizae with alternating light and dark bands. These mycorrhizae may also appear slightly beaded due to frequent constrictions. Both appearances are due to the successive occurrence of metacutization layers which encircle the meristem during periods of inactivity. Older living mycorrhizae are distinguishable by their white or translucent apices which contrast sharply with the brown color behind the apex. These white and translucent apices are minute and relatively inconspicuous in dormant mycorrhizae and become much longer and more conspicuous during periods of active growth. The mycorrhizae in fig. 1B are in their first period of active growth and consequently show no evidences of cyclic growth activity.

The longevity and number of ectendomycorrhizae vary greatly from seedling to seedling, from root to root on a given seedling and from one position to another along the seasonal increment of a given root. In 3-year-old seedlings, first order laterals with abundant mycorrhizae frequently have them clustered at the ends rather than the mid-portion of a seasonal increment. Mycorrhizae formed during one seasonal increment usually culminate their development in the following season and subsequently decline. Few mycorrhizae appear to survive for a third season.

Differences in External Appearance between Ectendomycorrhizae and Ectomycorrhizae

Ectomycorrhizae are more variable in appearance than ectendotrophic, and representative examples are shown in figure 1, C & D. These conform to most ectomycorrhizae in possessing conspicuous fungal mantles. The mycorrhizae in figure 1C have a smooth mantle without projecting hyphae, and those in figure 1D have a conspicuous white mantle with interconnecting rhizomorphs. Both of these are common in red pine plantations along with numerous other recognizable types. Variations occur in the length and frequency of dichotomous branches, the thickness and color of the fungus mantle and the extensiveness of the hyphal ramifications beyond the mycorrhizal surface. In most cases the ectomycorrhizae have conspicuous mantles and are distinguishable from ectendomycorrhizae in which the mantle is rarely visible to the naked eye. An exception occurs in the type of ectomycorrhiza with a smooth, colorless mantle as shown in figure 1C. An examination of these mycorrhizae with a hand lens will often reveal the mantle and the swollen appearance characteristic of these ectotrophic forms. However, an unequivocal separation of similar ectendomycorrhizae and ectomycorrhizae requires microscopic examination.

Anatomical Features of Ectendomycorrhizae

Figure 2B shows the appearance of an ectendomycorrhiza in medial longisection. The fungal mantle is relatively inconspicuous, a Hartig net of thick hyphae surrounds the cortical cells, and coarse intracellular hyphae occupy the cortical cells. These features are seldom uniform throughout the axis of a mycorrhiza. The intercellular hyphae extend acropetally in advance of the intracellular hyphae, and both tend to disappear in the basal portion of older mycorrhizae along with the senescence of the cortex. The fungi do not penetrate the endodermis and do not extend into the meristem. The barrier to their penetration of the meristem is not readily apparent during periods of root activity, but during periods of dormancy the fungi are barred from the meristem by the encircling metacutization layers. These layers are most strikingly demonstrated by fluorescence microscopy (Wilcox, 1968).

Ectendomycorrhizal Fungi in Long-Roots

The cortex of a long-root is also occupied by ectendomycorrhizal fungi. In the larger roots the fungi may remain discrete in the intercellular spaces, not forming a Hartig net, or may only form a rudimentary net (fig. 2A). However, the continuum of root diameters is marked by a continuum in the expression of infection symptoms. Progressively narrower roots display fungal features which increasingly resemble those in typical mycorrhizae (fig. 2C & D).

Some of these may possess rudimentary mantles and have their cortical cells widely separated by a Hartig net (fig. 2C). Figure 2C is from a second-order lateral, but in some cases it is difficult to assign a long- or short-root designation. It is not clear at what point such roots meet the definition of an ectendomycorrhiza as defined by Mikola (1965). According to him, ectendomycorrhizae are short-roots with a Hartig net and intracellular hyphae in the cortex.

Changes in infection occur over short axial distances in the root. The fungus advances in the growing root and also progressively dies out in the basal portions. Nevertheless the fungus does not always show a strict acropetal developmental sequence in the long-root. The fungus may vary both in radial and tangential distribution within a cross section of root and may also vary in distribution from proximal to distal ends of the seasonal growth increments.

Ectendomycorrhizal fungi are plentiful in mother roots up to 1.2 mm in diameter. With increase in size beyond this, the ectendotrophic hyphae gradually die out. The fungus tends to be absent in roots greater than 2.0 mm, but such roots are infrequent in the nursery. Even though the fungus may be sparse in a long-root, it may become concentrated in the vicinity of an emerging lateral. The fungi appear to prosper in direct proportion to their proximity to the meristematic centers of the root. The occurrence of the ectendomycorrhizal fungus in smaller roots may be related to the fact that the meristem in such a root is not moving away from the fungus at a rate too rapid for it to keep up. During cycles of elongation the meristem moves away but such cycles are briefer in small diameter roots and the fungus can again overtake the meristem. The fungus continues to extend acropetally in the first and second

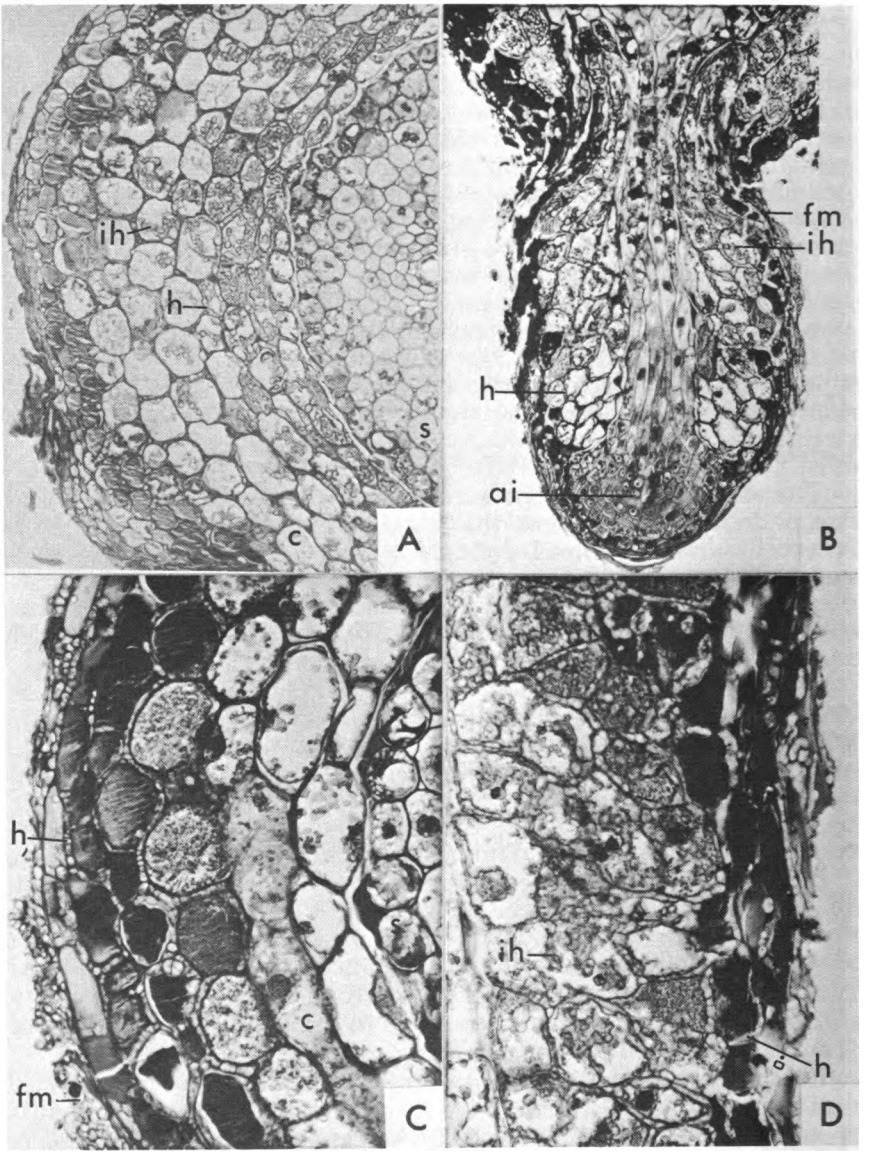

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Figure 2.—Anatomical details of ectendomycorrhizal fungi in red pine roots: *ai*, apical initials; *c*, cortex; *fm*, fungal mantle; *h*, Hartig net; *ih*, intracellular hyphae; *s*, stele.—A. First order long-root of 2-year-old seedling with ectendomycorrhizal infection in the inner cortex, 115X.—B. Ectendomycorrhiza emerging from third order long-root of 2-year-old seedling. Infection is continuous with that in the mother root, 100X.—C. Second order long-root of 2-year-old seedling with mantle and coarse Hartig net formed by ectendomycorrhizal fungus. Intracellular hyphae are absent at this level but occur about 50 μ proximally, 240X.—D. Small diameter first order long-root of 2-year-old seedling with heavy intercellular and intracellular infection and a weak mantle, 240X.

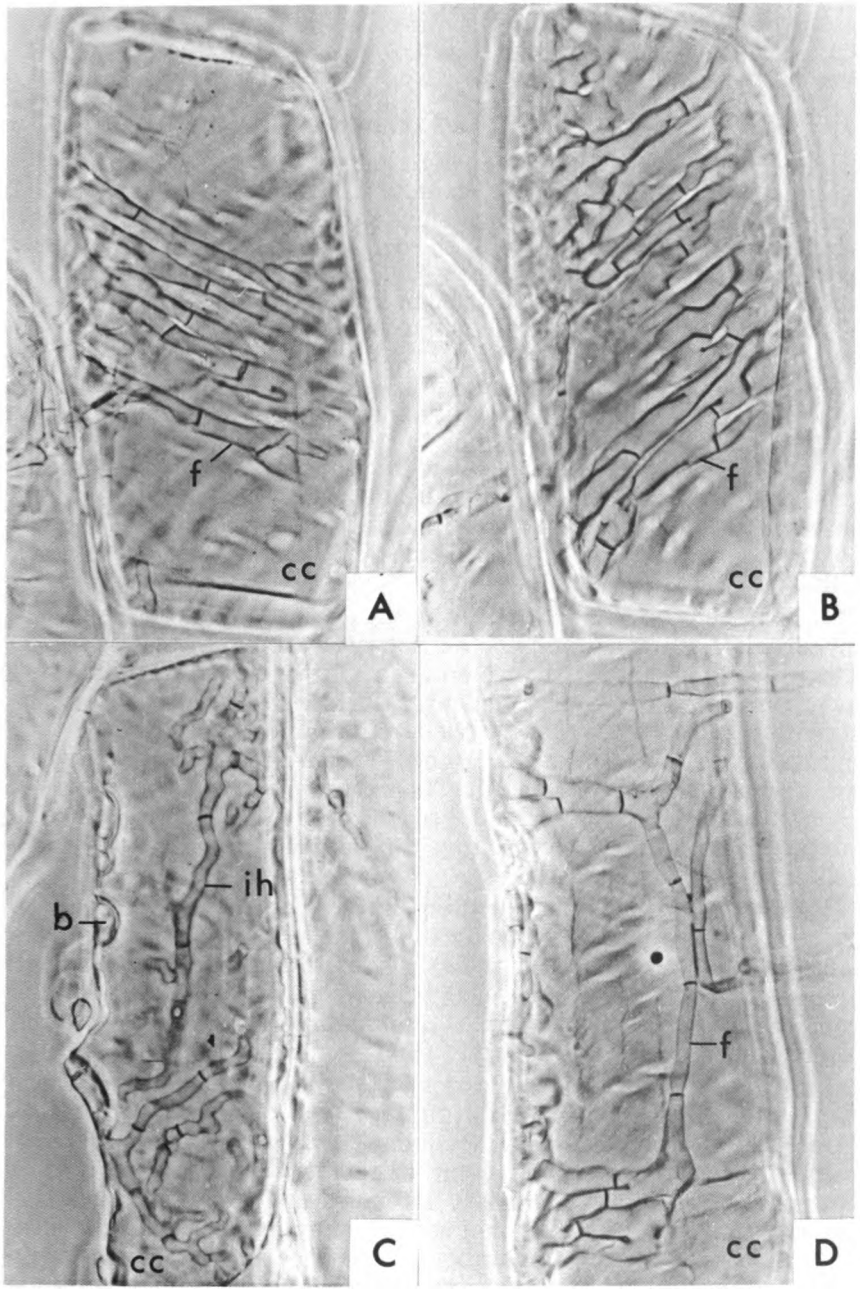

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Figure 3.—Cortical cells from macerated long-roots of 2-year-old red pine seedlings with associated hyphae of ectendomycorrhizal fungus: *b*, embedded fungal hypha; *cc*, cortical cell; *f*, fungal hyphae. 700X.—A. Upper facing wall of cortical cell with attached hyphae from intercellular net.—B. Lower facing wall of the same cell as A. The fungus spirals around the cell following the angle of the fibrils and the pit axes.—C. Intracellular hyphae. Indentations in wall result from encirclement of cell by intercellular hyphae prior to completion of cell enlargement.—D. Relationship between longtitudinal hyphae and encircling hyphal branches from intercellular network.

order laterals of 2–0 nursery seedlings after the roots have become dormant. In the roots of medium diameter, the fungus readily reaches the metacutization layers.

Hyphal Characteristics of the Ectendomycorrhizal Fungus

The hyphae vary from 3–10 μ in diameter with septae from 10–30 μ apart. The fungus grows acropetally in the intercellular spaces of the cortex and along the root surface. The longitudinal hyphae branch laterally in contact with the cortical cell walls(fig. 3D) and extend spirally around the cells. The spiral parallels the fibrillar angle of the cortical cell walls. The nature of the spiral network can be seen clearly by focussing the microscope alternately on the front and back walls of a macerated cortical cell (fig. 3A&B).

The spiralling hyphae penetrate the cell walls through pit areas as well as from appressorium-like structures (fig. 4C). Once inside the cell, the hyphae may continue to spiral around the inside of the cell wall. Haustorial branches grow into the cell interior and branch repeatedly to form an intricate, beaded and articulated ramification within the cell. The rounded outlines of this cellular structure contrast with the angular appearance of the intercellular network (fig. 3C).

In long-roots below 0.8 mm in diameter, the hyphae of ectendomycorrhizal fungi extend to the meristem, often surrounding it. The spiral network may develop around cortical cells before they have completed their enlargement. Such cells may later show constrictions where the hyphae have became embedded in the expanding wall (fig. 3C). The lateral branches of the intercellular network are frequently closely spaced. The resultant hyphae are tightly packed against each other and, because of the frequent septae, the surface view of the cell wall gives the appearance of a polygonal cellular network (fig. 3A&B).

Interspersed with the spiralling hyphae are the numerous appressoria-like bodies previously mentioned. These are frequently lobed in bizarre patterns (fig. 4B). The individual cortical cells are frequently completely covered by a complex intercellular network consisting of the longitudinal hyphae, spiralling strands, and appressoria-like structures (fig. 4D).

In addition to the characteristics of the intercellular network, the hyphae of ectendomycorrhizal fungi have other distinctive features. The individual hyphae frequently change in diameter and a large hypha may fork to form two smaller stands (fig. 4C). Branches often diverge and may extend radially as well as longitudinally. Individual longitudinal strands may extend along the surface of a long-root considerably in advance of the intercellular hyphae. One odd characteristic frequently noted was the presence of one hypha growing inside another (fig. 5A). This appears to represent some form of autolysis, but its significance is not understood.

The complex intercellular patterns seen in the long-roots are duplicated in the mycorrhizae arising from these roots. The cortical cells are smaller and the hyphal strands are also smaller in diam-

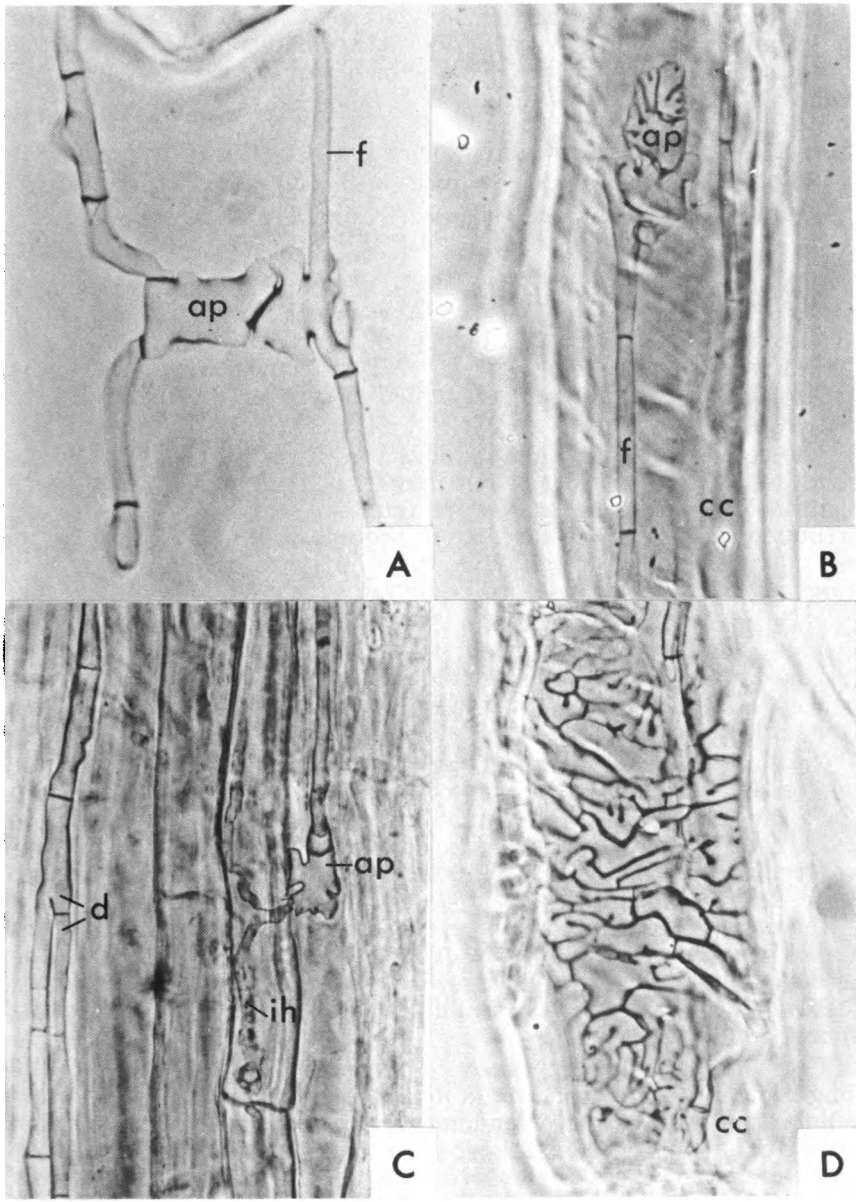

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Figure 4.—Details of hyphae of ectendomycorrhizal fungi in long-roots of 2-year-old red pine seedlings: *ap*, appressorium-like structure; *cc*, cortical cell; *d*, fungal dichotomy; *ih*, intracellular hypha.—A. Appressoria-like bodies on longitudinally extending intercellular hyphae, 980X.—B. Appressorium-like body on intercellular hypha, with intricately lobed structure, 740X.—C. Appressorium-like structure with penetration of hypha into adjoining cell and typical dichotomous branching of ectendomycorrhizal fungus, 370X.—D. Complex intercellular network consisting of longitudinal hyphae, spiralling strands, and appressoria-like structures, 670X.

eter. Maceration of these mycorrhizae confirmed the existence of longitudinal hyphal strands, encircling bands of hyphae, and appressoria-like structures. These form intricate patterns on the walls of the cortical cells (fig. 5C & D).

Initiation of Ectendomycorrhizae from Ectendomycorrhizal Mother Roots and the Secondary Invasion of Developing Ectendomycorrhizae by Ectomycorrhizal Fungal Symbionts

The initiation of ectendomycorrhizae from long-roots varies with the cyclic activity of the long-root as described in my earlier paper (Wilcox, 1968). If the ectendomycorrhizal fungus is present at the site of initiation, the emerging laterals are covered by the fungus except for an encircling band immediately over the meristematic region.

The ectendomycorrhizal symbiont was found in nearly all long-roots of nursery seedlings. Seedlings up to 4 years of age were examined. Ectomycorrhizae were found to occur with increasing frequency after the second year in the nursery, but the hyphae of ectendomycorrhizal fungi were still the initial colonizers of the long-roots in 4-year-old seedlings as well as in all younger seedlings.

The first mycorrhizae arising behind the meristem were recognizable as ectendomycorrhizae according to the anatomical features just described. However, in the older seedlings, an increasing number of these ectendomycorrhizae were secondarily invaded by other fungi and became converted to ectomycorrhizae.

One of the most common fungi acting as a secondary invader possessed a fine, non-septate mycelium. The mycelium had a diamter of approximately 1 μ and had closely spaced nuclei. The individual hyphae had widely spaced, diverging branches which appeared to grow straight in whatever direction they happened to arise. The preponderance of hyphae formed an interlacing network without obvious pattern. However, some directional strands did arise from those hyphae which followed the large hyphae of the ectendomycorrhizal fungus (fig. 5B). These strands formed long festoons which wove back and forth across the root connecting short-root branches.

These fine hyphae appeared to remain on the surface of the long-root and not to follow the hyphal strands forming the intercellular network of the ectendomycorrhizal fungus. These hyphae were found on occasional long-roots of the 2-year-old seedlings and on many of those of the 4-year-old seedlings. The fungus grew acropetally along the surface of the long-root behind the advancing ectendomycorrhizal fungus. The emerging laterals were often invaded shortly after the root had emerged and after it had been initially colonized by the ectendomycorrhizal fungus.

Many of the mycorrhizal branches continued to be occupied by both fungi. However, the ectomycorrhizal fungus frequently overran the ectendomycorrhizal one. In these cases, the ectendomycorrhizal fungus seemed to disappear, but maceration of many of these mycorrhizae which appeared to be completely ectomycorrhizal with the finer mycelium revealed the presence of some hyphae of the

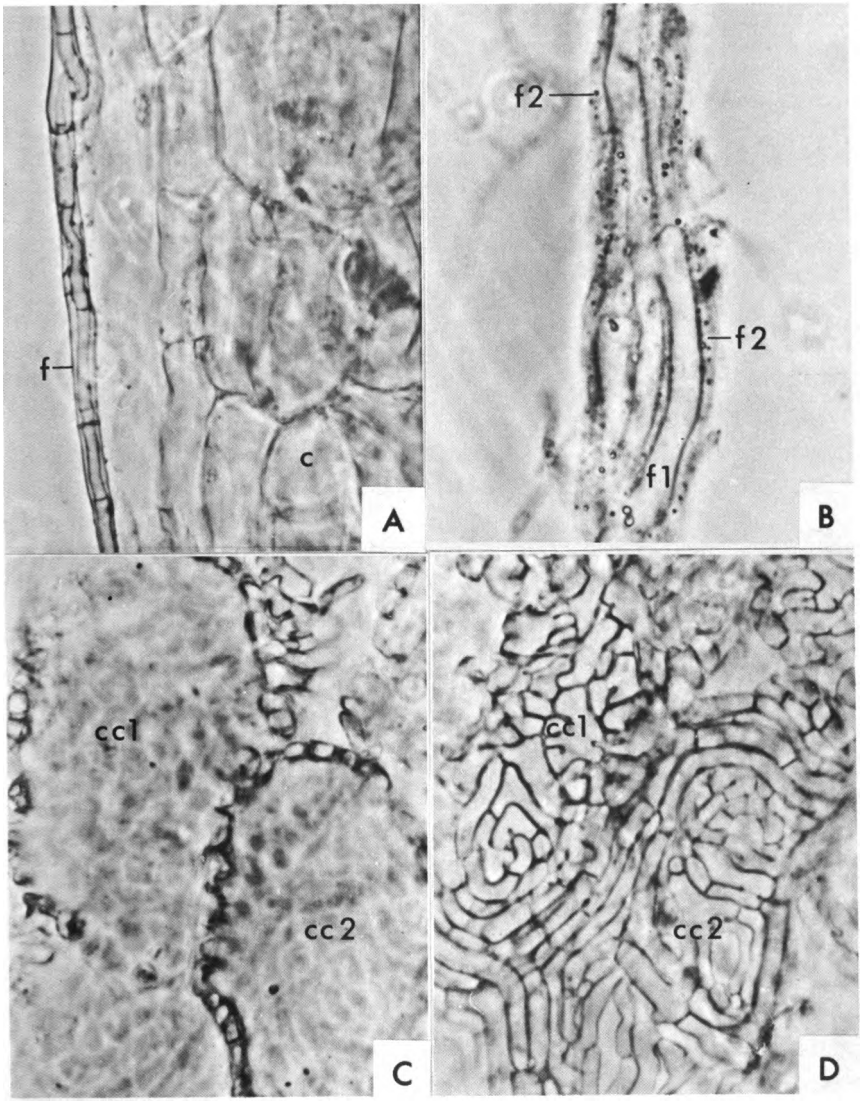

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Figure 5.—Additional structural details of ectendomycorrhizal fungus: c, cortex; *f*, fungus; *f1*, ectendomycorrhizal hyphae; *f2*, ectomycorrhizal hyphae; *cc1* and *cc2*, individual cortical cells.—A. Apparent autolysis of hypha, which is a frequent characteristic of ectendomycorrhizal infection, 330X.—B. Hyphae of ectendomycorrhizal fungus surrounded by small diameter, multinucleate, aseptate fungus that produces an ectomycorrhiza, approximately 1300X.—C. & D. Surface views of cortical cells from an ectendomycorrhiza. Microscope is focused below the Hartig net in C. to show outline of the cells. Details of the Hartig net over these cells are shown in D., 1300X.

ectendomycorrhizal fungus. Many of the larger dichotomous mycorrhizae showed an ectendomycorrhizal mycelium in the base and an ectomycorrhizal mycelium in the apex.

This particular ectomycorrhizal fungus rarely occupied an extended portion of the longitudinal growth increment of the long-root. A series of short-roots was invaded by the ectomycorrhizal mycelium with ectendomycorrhizae both proximal and distal to this series.

Although I have chosen to interpret the small hyphae as belonging to another fungus, there is a remote possibility that it represents a phase change of the ectendomycorrhizal fungus. This possibility should be investigated by pure-culture mycorrhizal synthesis. Since the ectendomycorrhizal fungus is found in the distal portions of most long-roots, it should be easy to isolate without contamination.

Anatomical Features of Aseptically-Grown Seedlings and of Naturally-Grown Non-Infected Roots

It should be emphasized that fungal infection does not alter the basic structure of the stele as suggested by earlier investigators. Figure 6A shows the transverse section of a short-root grown in aseptic culture. The cortex of such a root appears to be short lived (fig. 6B). Both ectomycorrhizae and ectendomycorrhizae appear to conserve cortical cells in the short-roots and also in the long-roots. The early senescence of the cortex of a naturally-occurring non-infected short-root is shown in figure 6C.

Anatomical Features of Ectomycorrhizae in Comparison to Ectendomycorrhizae

I have implied that, although the two types of mycorrhizae cannot always be distinguished externally, it is possible to do so microscopically. This generalization requires some explanation.

The ectomycorrhizae are obviously caused by a variety of fungal species rather than by a single species as in the ectendomycorrhizal type. Consequently, the anatomical features of the former show a much greater variation than the latter. Some of the variations in anatomy are shown in figure 7. Although these photomicrographs are of plantation and flask culture material, similar ectomycorrhizae were obtained from the nursery.

The ectomycorrhizae possess a mantle which varies from thin to voluminous. The Hartig net may be of uniform thickness throughout the cortex or may be much thicker between the outer cortical cells than in the deeper layers. The net may penetrate to the endodermis, half way, or only through the outer layer. In some cases there may be a voluminous mantle with no apparent intercellular penetration. Figure 7D is an ectomycorrhiza with both a well developed net and a thick mantle from a plantation red pine. The mycorrhiza formed by the fungus with the small diameter described earlier corresponded to the type shown in figure 7C.

The axial distribution of the Hartig net in ectomycorrhizae has analagous discontinuities and developmental patterns to those of ectendomycorrhizae. Intracellular hyphae occur sporadically, but these are small in diameter and sparsely branched.

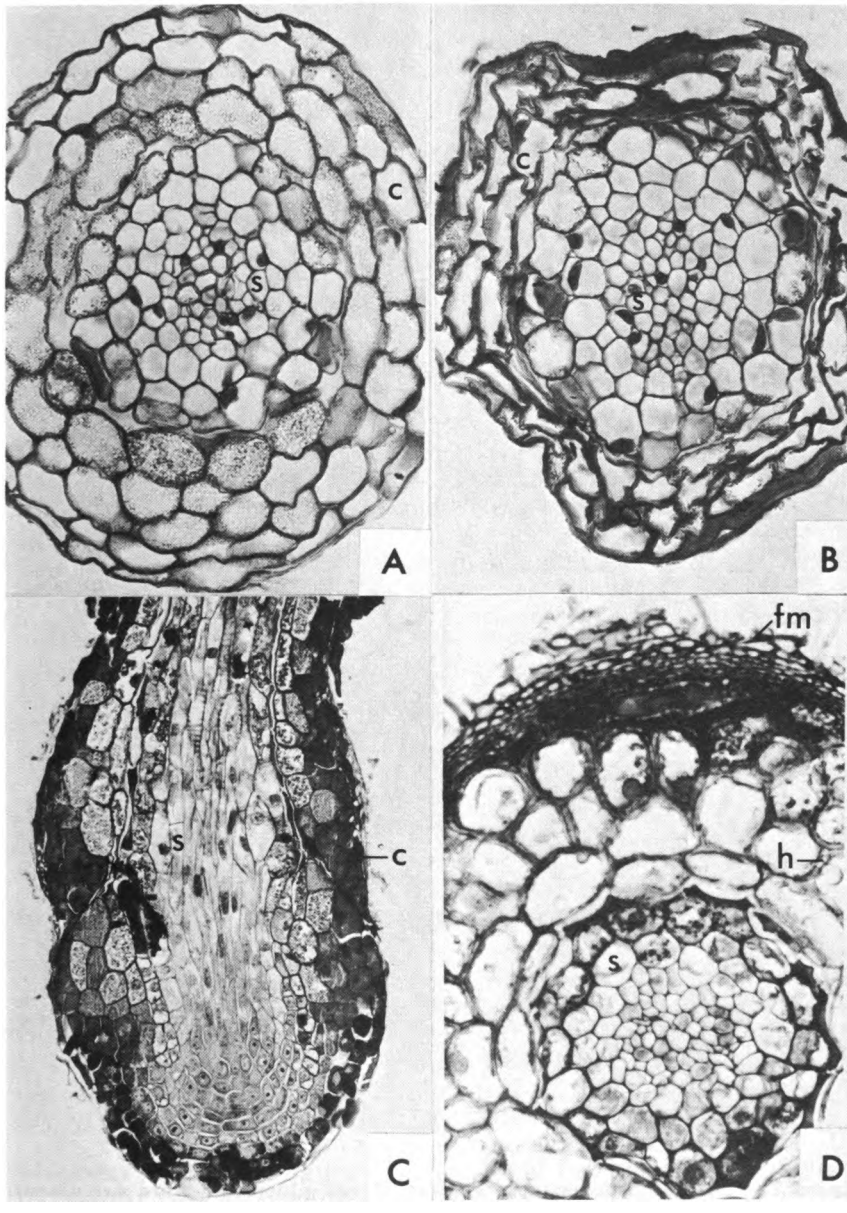

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Figure 6.—Uninfected short-roots and changes induced by ectomycorrhizal fungus: *c*, cortex; *fm*, fungal mantle; *h*, Hartig net; *s*, stele.—A. Short-root from 1-year-old seedling grown in aseptic flask culture. Section is in proximity to meristem of recently-formed short-root, 250X.—B. Early collapse of cortex in uninfected short-root from a similar flask culture as A, 260X.—C. Longisection of uninfected short-root of a 2-year-old nursery seedling. Early senescence of cortical tissue is characteristic of such roots, 120X.—D. Ectomycorrhiza with black mantle of thick-walled hyphae, which is common in red pine plantations and occurs with increasing frequency in nursery seedlings 3 years of age and older, 235X.

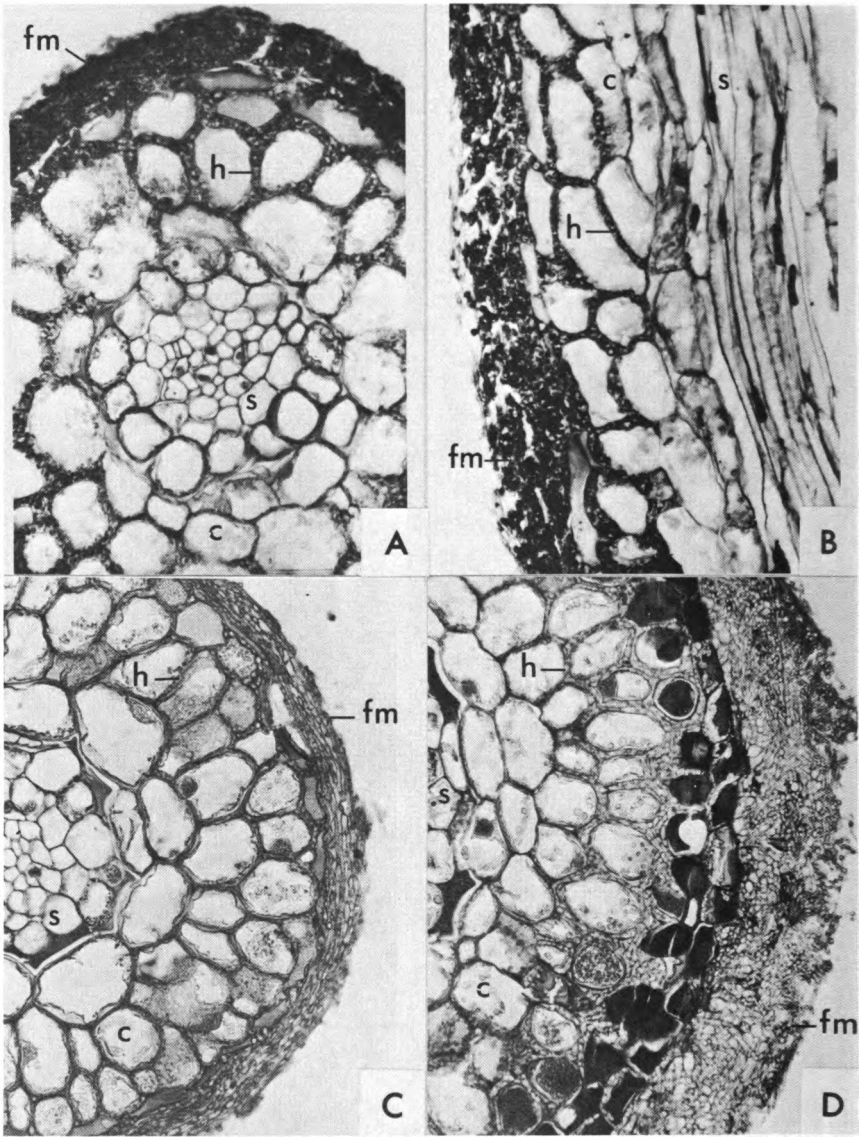

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Figure 7.—Characteristic ectomycorrhizae of red pine with more prominent mantles and finer-textured Hartig net than ectendomycorrhizae: *c*, cortex; *fm*, fungal mantle; *h*, Hartig net; *s*, stele.—A. Transverse section of mycorrhizae formed in pure culture with *Amanita muscaria*, 235X.—B. Longisection of mycorrhiza with *Amanita muscaria*, 235X.—C. Ectomycorrhiza with relatively thin mantle and inconspicuous Hartig net, 235X.—D. Ectomycorrhiza with relatively thick mantle and prominent Hartig net, 210X.

In some ectomycorrhizae, the outer cortical cells are radially elongated with a consequent hypertrophy (fig. 7C). Other ectomycorrhizae and ectendomycorrhizae do not display cortical hypertrophy. The various black mantled mycorrhizae are particularly distinctive (fig. 6D).

It is not known how distinctive each ectomycorrhiza is in respect to its particular fungal symbiont. Figures 7A & B are of the ectomycorrhiza synthesized from *Amanita muscaria* which shows a number of anatomical features differing from any of those found in the plantations during the investigation. The *Amanita*-red pine mycorrhiza has a mantle 70–150 μ thick, composed of a pseudoparenchymatous tissue with chromophilic spherical inclusions. The Hartig net is conspicuous, and epidermal and sub-epidermal cells are filled with a very dense, fine granular matrix. The long-roots are also lightly infected.

Ectomycorrhizal fungi colonize the long-roots of plantation seedlings in an analogous manner to the ectendomycorrhiza in the long-roots of nursery seedlings. The fungi are able to form an extensive net in the larger roots of 2.0 or more mm in diameter without any apparent inhibition of their elongation. However, a considerable number of small diameter subordinate mother roots in the plantations remain fungus free in contrast to the small diameter nursery roots which are heavily filled with the ectendomycorrhizal fungus. It is possible that the small-diameter long-roots in the nursery are not inhibited in elongation by the ectendomycorrhiza while comparable subordinate mother roots are inhibited upon fungal invasion and become indistinguishable from short-roots.

The plantation long-roots do not conform to the ectomycorrhizal pattern in toto. Subordinate mother roots are occasionally encountered with ectendomycorrhizal fungal hyphae. Ectendomycorrhizae also occur.

Prevalence of Ectomycorrhizae in Nursery Seedlings

Other ectomycorrhizal species occur besides the secondary invader described earlier. Ectomycorrhizae similar to the types found in plantations appear to gradually invade the nursery when the seedlings are permitted to remain beyond two years. They are less frequent than the type formed by the fungus with thin hyphae described above, and their development in relation to the ectendomycorrhizal fungus remains to be studied.

An ectomycorrhiza with the same anatomical features as the black mycorrhiza in figure 6D was found in several of the 2-year-old seedlings. Another form was also encountered with the characteristics shown in figure 7C, except for larger-diameter hyphae. Thus far, in the nursery I have not found ectomycorrhizae with the conspicuous outer mantle of figure 1D or with the exaggerated anatomical features of figure 7D.

Conclusions

The preceding observations indicate that the characteristics of ectendomycorrhizae are unique and are very likely caused by a single fungus. These features are similar in all red pine seedlings,

and in roots of the same root class, the patterns of mycorrhizal development are similar in the way they vary from proximal to distal ends of the seasonal increments. In all roots, dormancy, cyclic growth behavior of roots, and colonization of long-roots by fungi are key factors in the development of mycorrhizae.

A study of the increasing frequency of ectomycorrhizae in older nursery seedlings showed that a type with thin aseptate hyphae was due to the secondary invasion of short-roots which had already started to develop as ectendomycorrhizae. Although this type was most common, the occurrence of other ectotrophic types indicated the later invasion of other fungal species. Despite the occurrence of these distinctively ectotrophic types, it is not clear that the ectendomycorrhizal fungus dies out following subsequent invasions. It is possible that it continues to colonize the distal portions of long-roots, but further investigations are necessary to determine this.

The above observations indicate that the fungal colonization of long-roots and the interrelations between different fungi in the long-root are as important to the development of mycorrhizae as is the invasion of short-roots. In fact, anatomical studies limited to mycorrhizal short-roots provide limited understanding of mycorrhizal development. A better appreciation of the mycorrhizal habit is obtained by a consideration of the root system as an entity.

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6.

Some Aspects of Tree Root Distribution

M. V. Bilan

Survival and productive potential of a tree are determined to a great extent by its root system. Roots provide anchorage, water, and nutrients needed for growth and development. Variation among the root systems of forest trees and the importance of this knowledge in the practice of forestry were stressed as early as the beginning of 19th century (Hartig, 1808; Cotta, 1835). Growth and development of root systems are affected by both heredity and environment. While the initial type of root growth in most tree species is determined primarily by heredity, the subsequent development of root systems is more or less dominated by the environment.

The main objective of this paper is to outline what is known about gross patterns of rooting in trees and their relationship to hereditary and environmental factors.

Inherent Root Characteristics

Root Habit

When grown under favorable environmental conditions, seedlings of individual tree species develop distinctive root systems which with some modification persist through subsequent stages of development. Thus, root systems of individual tree species are characterized by a definite root habit and root intensity. Root habit of a species pertains to the form, direction, and distribution of the large roots within the root system; root intensity denotes the form, number, and distribution of the small roots.

Büsgen and Münch (1927) divided the root systems of European forest tree species into three basic habits (Wurzeltrachts): taproot, heartroot, and flatroot. With a slight modification, this general classification of root habits is still in use. Köstler et al. (1968) divided the tree root systems into the taproot, heartroot, and sinkerroot habits (Grundtypen). Taproot habit is characterized by a strong dominance of a downward growing main root which may branch to a greater or lesser degree. This habit often develops in *Carya, Juglans, Quercus, Pinus,* and *Abies.* Absence of a strong taproot and the presence of numerous strong roots radiating diagonally from the base of the tree are the main characteristics of a heartroot habit. This root habit is found in *Larix, Betula, Carpinus,* and *Tilia.* The sinkerroot habit is dominated by strong laterals from which heavy vertical "sinkers" tend to grow straight downward. This root habit is common in *Picea, Fraxinus,* and *Populus.*

Because of the multitude of species and scarcity of information on root systems of older trees, no generally accepted classification of root habits of North American trees is available. Initial root

systems of woody plants are frequently divided (Toumey and Korstian, 1947) into three classes: 1) species with long taproot and without prominent laterals; 2) species with short initial taproot and prominent laterals; and 3) species with long initial taproot and prominent laterals.

The deeply penetrating, juvenile root without prominent laterals is characteristic of species adapted to the regions where the surface layers of soil get excessively dry during the growing season. Development of a taproot in these species proceeds at the expense of the energy stored in the seed and reduced growth of the shoot. The initial root in singleleaf piñon (*Pinus macrophylla* Torr. and Fremont) may be up to ten inches long before the cotyledons push out from the seed coat (Toumey and Korstian, 1947). The same authors reported that one-year-old sugar pine (*P. lambertiana* Dougl.) may penetrate up to 26 inches deep, while the shoot is only two to three inches long. The classic example of excessive taproot development at the prolonged expense of shoot growth is the grass stage of longleaf pine (*P. palustris* Mill.). While the stem may be only one to two inches high, the carrot-like main root may be one inch in diameter and several feet long.

Species with short initial taproot and prominent laterals are adapted to the shallow soils where moisture is not a limiting factor. Here belong species of *Picea, Tsuga, Taxodium, Libocedrus, Betula, Carpinus* and *Cornus.*

Species with long initial taproots and prominent laterals have deeply penetrating and wide-spreading root systems, and consequently, they require deep and moist soils for their optimum development. Representatives of this group are genera *Juglans, Carya, Liriodendron, Liquidambar* and some species of *Pinus* and *Abies.* The main root of one-year-old black walnut (*Juglans nigra* L.) can penetrate 35 inches deep, and some of the laterals may be 40 inches long (Toumey and Korstian, 1947). Loblolly pine (*Pinus taeda* L.) seedlings, grown for five months in transparent tubes filled with sandy loam soil, produced four-foot-long taproots and about 100 first order laterals per seedling. Most laterals stopped growing within a few weeks, but some grew continuously for 24 weeks, attaining lengths of 30 inches (Bilan, 1965 and 1967).

Some species have the capacity to adapt their juvenile root system to a variety of environments and, thus, can get established on dry as well as wet sites. According to Toumey (1929) red maple (*Acer rubrum* L.) has a very high root plasticity and produces shallow, wide-spread root systems on swampy sites and a deep taproot with weak laterals on dry sites. Box elder (*A. negundo* L.) is also known to have high root plasticity (Biswell, 1935).

Juvenile root habit in some species is so rigid that it is also retained in the advanced age. Wahlenberg (1946) reported on a 250-year-old longleaf pine growing in deep sand with a shaft-like taproot larger in diameter one foot below the soil surface than the stem diameter at breast height. Diameter of eighty-year-old European silver fir trees (*Abies alba* L.) were reported to be larger at 15 cm below the ground than one meter above the ground (Safar, 1954). A strong development of taproot in 42-year-old pignut hickory (*Carya glabra* (Mill.) Sweet) was reported by Stout (1956).

Initial root habit of a tree species is indicative of its natural habitat. Thus, species with long initial taproot are adaptable to deep arid soils, while species with a short initial taproot and long laterals are at home on shallow moist soils. A deep initial root system combined with shade tolerance is a very good indicator of success in competition. High root plasticity and shade tolerance are probably responsible for an extremely wide natural range of the red maple.

Root Intensity

While a root habit indicates the volume of soil occupied by a root system, the form, number, and distribution of the small roots determine the intensity with which the occupied soil volume is tapped. Büsgen (1901) was the first one to distinguish between the intensive and extensive types of root systems. He considered an intensive root system one where a smaller soil volume was penetrated by many roots and an extensive system where greater soil volume was penetrated by fewer roots. Köstler et al. (1968) generalized that conifers have extensive and broadleaves have the intensive root systems, but some overlapping exists between these two groups. For example, *Larix* and *Pseudotsuga* are conifers with relatively intensive root systems, while *Quercus* and *Ulmus* are the broadleaves with relatively extensive root systems. *Acer*, *Fagus*, and *Carpinus* are considered as tree species with the most intensive root systems.

Among North American trees, genus *Pinus* seems to have the least branching root system. Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) were found to have more profuse fine roots than western white pine (*Pinus monticola* Dougl.) (McMinn, 1956). According to Leaphart (1958), western hemlock, grand fir (*Abies grandis* (Dougl.) Lindl.), and Englemann spruce (*Picea engelmannii* Parry had four times as many root tips per centimeter of parent lateral root in the 1-15 mm diameter class than the western white pine. Berndt and Gibbons (1958) reported that Douglas-fir and quaking aspen (*Populus tremuloides* Michx) had more concentrated masses of roots than ponderosa pine (*Pinus ponderosa* Laws). According to Kozlowski and Schlotes (1948), six-month-old flowering dogwood (*Cornus florida* L.) had three and one-half times as many roots as loblolly pine of the same age. Loblolly had only two orders of root branching, while dogwood had five. This author observed that, at least in young trees, the proliferation of lateral roots of four major southern pines is inversely related to the dominance of the main root: thus, it is strongest in shortleaf pine (*Pinus echinata*), followed in descending order by loblolly, slash (*P. elliottii*), and longleaf.

Sugar maple (*Acer saccharum* Mush.) and yellow birch are considered to be species with intensive root systems (Fayle, 1965), as is red maple (*Acer rubrum* L.) (Lyford and Wilson, 1964), but there is not enough information available to classify the North American broadleaves on the basis of their root intensity.

Intra-Species Variations of Root Systems

Racial variation within many commercial forest tree species has been studied, but mostly with emphasis on growth and development

of shoots rather than the root systems. Advanced root systems are expensive to study, and are so modified by the environment that it is hard to distinguish inherent characteristics. Since most variation studies use seedling material, even the new literature provides very little information about intraspecific root variation in older trees.

Engler (1905) observed that two-year-old Norway-spruce (*Picea abies* L.) seedlings from a high elevation seed source had larger root systems than the comparable seedlings from a lowland source. The above differences could not be detected 22 years later (Nageli, 1932). Scots pine of Prussian provenance at age 28 showed better stem form than pine of Hessian provenance and also tended to develop more definite taproots (Biebelriether, 1964). Differences in root development among provenances of European larch (*Larix decidua* L.) and Japanese larch (*Larix leptolepis* (Sieb. and Zucc.) Gord.) were reported by Leibundgut and Dafis (1964) and Barner (1962).

Clonal differences in root characteristics have been reported for red maple (Snow, 1939), sugar maple (Dun and Townsend, 1954), several *Populus* species (Bloomberg, 1963, Wilcox and Farmer, 1967), and yellow-poplar (*Liriodendron tulipifera* L.) (Steinbeck and Kormanik, 1968).

Wakeley (1953) reported that in 1928 longleaf pine seed collected somewhere in southern Georgia yielded seedlings strikingly more diffuse-rooted than those from other sources. Also Snyder (1961) stated that roots of longleaf pine seedlings from eastern Georgia seed appeared more fibrous and had significantly more lateral roots than those from collections further west. Differences in initial root development seem to exist between two races of slash pines: *Pinus elliottii* Engelm. and *P. elliottii* var. *densa* Little and Dorman (Bethune, 1966 and Squillace, 1966). The author observed that among loblolly pine seedlings grown in tubes, there always were a few individuals which did not develop a strong taproot even after three years.

These are probably only a few of many inherent root differences due to provenance; others, more directly affecting mycorrhizae formation, are possible and should be looked for. More studies are needed dealing primarily with the species having extensive natural range and forming a variety of ecotypes.

Effect of Environment

As tree seedlings get older, their natural root habit tends to be increasingly modified by such environmental factors as physical and chemical soil properties; soil moisture, aeration, and temperature; and root competition. Roots which are located in favorable micro-environments grow and develop at a higher rate than those which have encountered adverse growing conditions.

Physical Soil Properties

Bedrock and hardpan mechanically impede root penetration and can modify even the strongest taproots. On thin skeletal soil in Colorado, Douglas-fir, ponderosa pine, and quaking aspen, all developed shallow root systems (Berndt and Gibbons, 1958). McMinn (1962) observed that taproot predominance in Douglas-fir was

more quickly obscured in shallow than in deep soil. When toproots reached bedrock, fragipan, or cobbles, they branched out and followed the contour of the obstruction. Wahlenberg (1946) described a 30-year-old longleaf pine with only a 1.5-foot taproot as a result of hardpan in Leon fine-sand.

Heavy soils limit vertical and horizontal root distribution as well as root elongation and branching. A slightly increased sand content in loamy soil doubled the length of horizontal roots and stimulated much finer root texture on two-year-old loblolly pines (Bilan, 1960). Seedlings growing in sandier soil had 25 percent of their root weight at a depth of 12 to 24 inches, as compared to 13 percent for seedlings growing in heavier soil. In an open field, over 70 percent of root weight of two-year-old loblolly pines was concentrated in the upper six inches of soil, which was much lighter in texture than the lower layers.

Root dry weight of two-year-old loblolly and shortleaf pine seedlings was less in Axtell sandy-clay loam than in three other coarse-textured soils. Roots in the Axtell soil were coarser, shorter, and less branched than in the remaining soils (Bilan and Stransky, 1966). Korstian and Coile (1938) reported that in a 31- to 35-year-old stand of loblolly pine, the A horizon had 10 times as many fine roots per square foot of the profile as did the B horizon.

Soil compacted in the process of planting can affect subsequent root distribution of the planted seedlings. It has been demonstrated that in both machine and bar-planted southern pines, many more roots are growing along the plane parallel to the slit than along the plane perpendicular to it (Bilan, 1960; Haines and Pritchett, 1965; Little and Somes, 1964). The author has observed that one-plane root elongation in bar-planted seedlings was more pronounced in heavy than in light soils.

Soil Moisture and Aeration

At favorable temperature and with adequate nutrients, root growth of trees is controlled primarily by soil moisture and aeration. Soil moisture affects root growth directly by controlling water supply and indirectly by affecting soil aeration.

The roots of all trees grow best in moist, well-aerated soils. Bilan (1960) reported that two-year-old loblolly pines, growing in scalped, mulched plots in East Texas, had eight-foot lateral roots growing in the upper three inches of soil (fig. 1). Many roots grew on the soil surface just under the layer of mulch where combination of moisture and aeration was most favorable. Roots of seedlings growing in scalped, unmulched plots, where soil surface was compacted and dry, were only two feet long and did not grow closer to the soil surface than two inches. Kozlowski (1949), has shown that large root systems develop in tree seedlings when grown in soil maintained close to field capacity, in contrast to sparse root development in soils allowed to dry almost to permanent wilting before rewatering. In well-aerated soil profiles, tree roots usually proliferate in the layers affording the greatest moisture supply (Brown and Lacate, 1961; Wilde, 1958; Gary, 1963).

Reed (1939) reported that the slowest period of root growth of loblolly and shortleaf pine seedlings in summer coincided with periods of the lowest soil moisture. The author observed that root

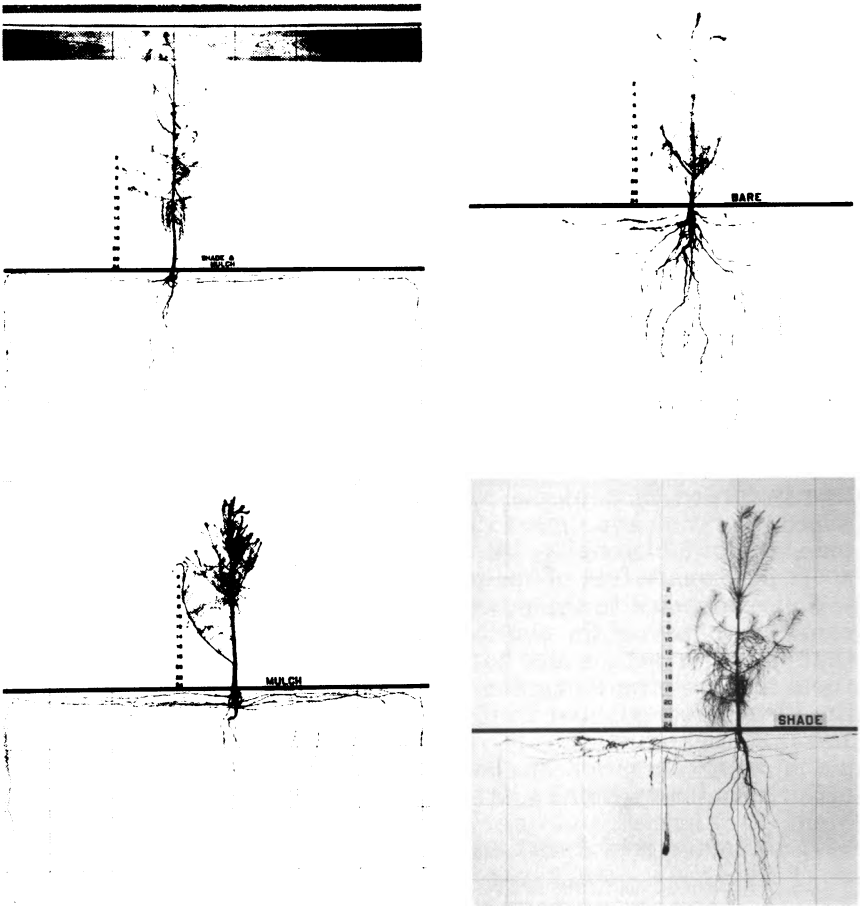

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Figure 1.—Root systems of two-year-old loblolly pines growing in an open scalped field in East Texas in bare, mulched, partially shaded, and partially shaded mulched plots.

growth of loblolly pine seedlings grown in tubes stopped completely long before the needle moisture fell to the critical level of 85 percent (expressed on oven-dry weight basis). Both the cessation of root elongation under moisture stress and root regeneration under improved moisture conditions proceeded from the root collar downward (Bilan and Jan, 1968).

Zahner (1968) discussed the effect of soil moisture on tree root development. He pointed out the difficulties in separating the root growth decline due to the deficient soil moisture *per se* from that due to the increased impedance of drying soil. It seems that some tree species have better adaptability to soil moisture regime than others. Steinbrenner and Rediske (1964) found that Douglas-fir seedlings had roots concentrated near the surface when soil was well watered, and roots penetrated deeply when surface soil moisture was not at optimum. Ponderosa pine, however, developed deep root systems regardless of soil moisture regime.

Water saturation of the soil results in a deficiency of oxygen and an accumulation of carbon dioxide. Such conditions result usually in reduced root growth and subsequent root mortality. Some species are capable of growing in water-saturated soils. They are usually swamp species such as baldcypress (*Taxodium distichum* (L.) Rich.) water tupelo (*Nyssa aquatica* L.), and some species of *Salix, Alnus,* and *Betula.* McQuilkin (1935) reported that pitch pine (*Pinus rigida* Mill.) is capable of extensive root growth in saturated soils below the water table. Southern white cedar (*Chamaecyparis thyoides* (L.) B.S.P.) grows usually in frequently inundated or saturated soils (Korstian and Bush, 1931).

Roots in swamps are relatively long and poorly branched. The author observed that roots of loblolly pine seedlings grown in wet and poorly aerated soil are black and string-like. Heikurainen (1964) observed that after draining swampy soils, roots of tree species increase their branching and form sinkers reaching greater depths.

Kramer (1949) stated that it seems unlikely that under field conditions any significant amount of root growth occurs at or near the permanent wilting percentage. He also concluded that excess of water and poor aeration of soil have at least as great an influence on the root systems of trees as does deficient soil moisture or heavy, impermeable soil strata.

Soil Temperature

In temperate regions, the beginning and end of the seasonal cycle of root growth are controlled largely by temperature. While temperature requirements vary among tree species, the minimum temperatures for root growth range from slightly above 0° to 7°C; the optimum, 10° to 30°C; and the maximum, 25° to 30°C (Lyr and Hoffman, 1967). Warm climate species begin and cease root growth at higher temperatures than species native to cooler climates.

During the dormant season, root growth of southern pines is controlled primarily by low temperatures. Reed (1939) reported slowest root growth in loblolly and shortleaf pines during periods of lowest soil temperature. Similar results were reported by Turner (1936) and Bilan (1961). Barney (1951) found that roots of loblolly pine seedlings in a controlled environment grew most rapidly at 20° to 25°C, while growth at 5° and 35°C was less than 10 percent of the maximum. Bilan (1967), observing root elongation of loblolly pine seedlings in tubes, found that, outdoors, roots elongated until average weekly minimum temperature fell below 34°F and at least one daily minimum was below 28°F. Roots resumed growth in the spring when none of the daily minima fell below 30°F. He found that roots in a growth chamber elongated during a 75°-35°F thermoperiod but stopped growth after 14 days of a 50°-35°F thermoperiod. Roots resumed growth within three days in a thermoperiod of 50°-40°F. This prompt resumption of growth after cessation caused by low temperature contrasts with several weeks before roots resumed growth after growth was stopped by moisture stress.

Lyford and Wilson (1966) built a shelter over the trays containing single exposed roots of several tree species growing in a natural environment. They found that day-to-day variation in growth

rate of red maple root tips was highly sensitive to temperature. The rate of root growth in unheated trays closely paralleled the variation in daily mean, outside air temperature, while the roots in heated trays grew essentially at constant rates.

Temperature also plays an important role in root regeneration on out-planted tree seedlings. Krugman and Stone (1966) reported that root regeneration of ponderosa pine planting stock was significantly increased after exposure to at least 90 cold (6°C or less) nights. Beyond this period, the root regeneration potential was clearly correlated with the number of cold nights to which the seedlings had been previously exposed. Low temperature controlled root regeneration of one-year-old loblolly pine seedlings out-planted in the field in East Texas in the middle of November. Bi-weekly excavations of seedlings revealed that, from the late November until the end of March, the percentage of growing root tips per seedling followed closely the fluctuation of minimum air temperature (Bilan, 1961).

Effect of temperature on root morphogenesis in Scots pine was reported by Slankis (1949). Roots of *Sequoia* seedlings appeared to be the healthiest at 18°C, while at 8°C they were short and thick (Hellmers, 1963). Roots of two-year-old loblolly pine seedlings growing in a field in bare soil, where soil surface temperatures were higher than in shaded and mulched plots, had short and stout roots with very short branches as compared with long and fibrous roots in mulched and shaded plots (fig. 1). Hardly any roots were found in the upper two-inch soil layer on scalped bare plots, while in scalped and partially shaded plots many roots grew close to the surface (Bilan, 1960).

It can be concluded that temperature affects all aspects of root growth and development in trees. The minimum, optimum, and maximum vary with the species, thermoperiod, photoperiod, and probably other environmental and inherent conditions.

Root Competition and Interaction

When plants grow close together, their root systems are smaller than when they grow further apart. Weaver and Kramer (1932) reported roots of tree seedlings which were greatly reduced by competition with prairie grasses. Coile (1940) concluded that the smaller root systems of forest-grown loblolly pine seedlings as compared with open-grown seedlings reflected more intense root competition. Vertical and horizontal root distribution of two-year-old loblolly pine seedlings growing in sod was about the same as that of seedlings growing in scalped bare soil, but the roots weighed only one fourth as much (fig. 2). The number of first order laterals and their degree of branching was much greater in scalped bare soil than in sod (Bilan, 1960).

Roots of spruce in mixture with beech and oaks, and Scots pine in mixture with beech are said to penetrate deeper than in pure stands (Köstler et al. 1968).

Roots of one species can either stimulate or inhibit the roots of other species. The effect can be either direct through the root grafts (Graham and Bormann, 1966) or indirect through the root exudation into soil. Uemura (1961) reported on a beneficial effect of *Alnus, Robinia* and *Acacia* upon growth of *Pinus densiflora.*

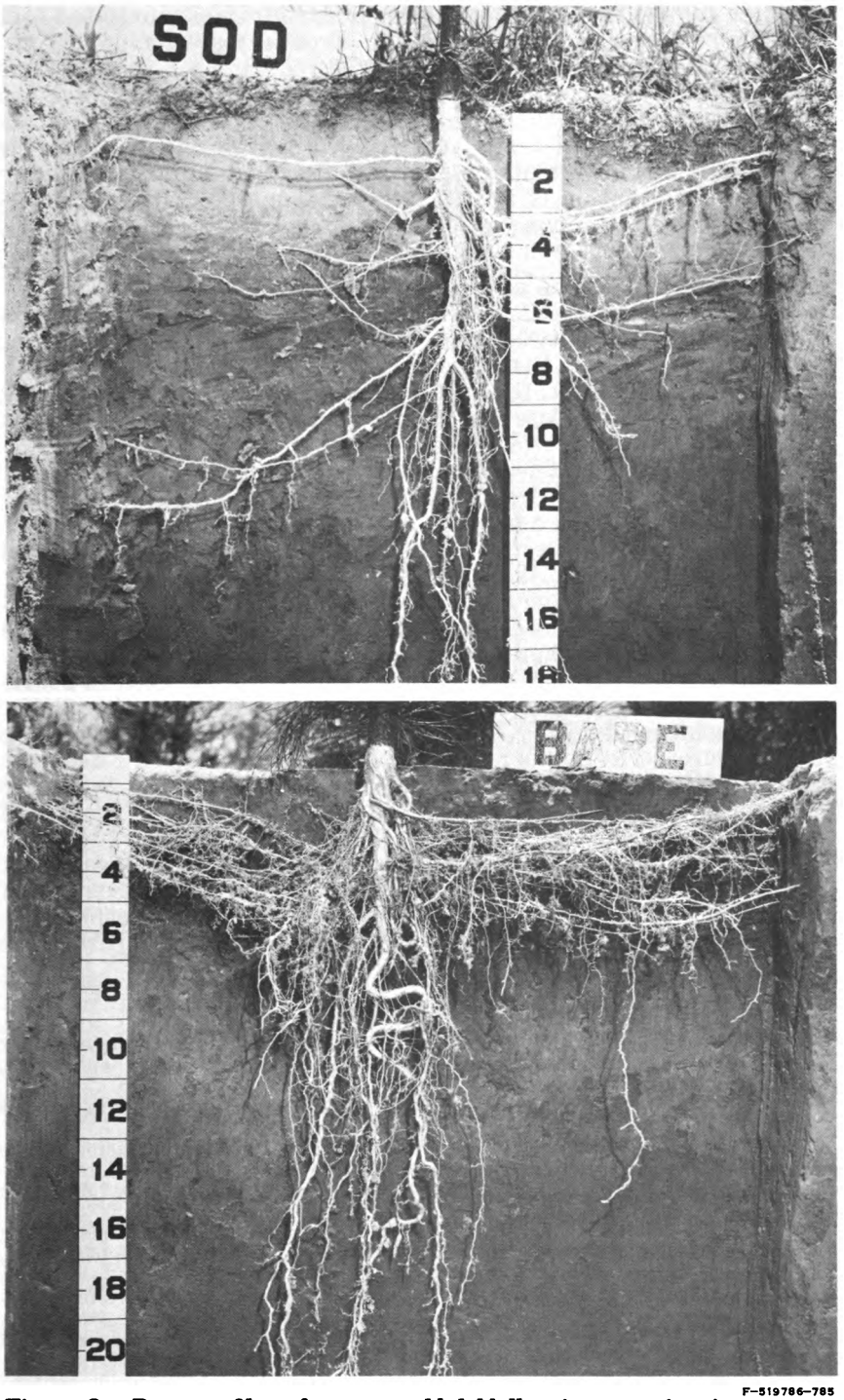

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Figure 2.—Root profiles of two-year-old loblolly pines growing in an open field in East Texas in sod and in scalped bare plots.

Lyubich (1958) reported that *Ulmus pinnato-ramosa* inhibited root systems of *Quercus pedunculata* and *Fraxinus viridis*.

It has been well documented that the reduction of root systems under intense competition is caused primarily by the diminished amount of available moisture and nutrients. It also has been established that growth of many tree species can be improved by the presence of legumes or other species having the ability of nitrogen fixation. Information is needed on root exudates and their direct and indirect effect upon soil chemistry, soil microorganisms, and the subsequent development of root systems in tree species.

General Implications of Root Distribution

Initial root systems of individual tree species are generally predetermined by heredity and are indicative of the natural edaphic environment in which these species thrive. Thus, strong dominance of the main root indicates deep and mostly dry soils, while weak main roots and long laterals indicate adaptability to shallow and moist soils. Since individual roots situated in a favorable microenvironment grow and develop better than those situated in adverse conditions, subsequent development of the root systems is governed primarily by the environment.

In addition to the vertical and horizontal extension of the root system, survival and productive potential of a tree species depends on the intensity with which it is able to tap the soil volume occupied by its root system. Thus, a profusely branching root system has a definite advantage over a system consisting of string-like, sparsely branching roots. Relatively small absorbing area in poorly branched root systems can be greatly enlarged by the abundance of mycorrhizae.

The increase in number of individuals per unit area intensifies the competition and usually reduces the root systems. Some species may actually benefit from being in mixed stands, while growth of others may be inhibited. Mutual effect can be direct through root grafting or indirect through the root exudation and modification of soil chemistry and/or soil microbiology. Better understanding of the actions and interactions taking place in the rhizosphere is necessary before we can maintain plant communities utilizing the sites to the fullest extent.

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Separate No. FS-287

7.

Ectomycorrhizae as Biological Deterrents to Pathogenic Root Infections

Donald H. Marx

Numerous investigators in the last several decades have shown that ectomycorrhizae are essential to the establishment and growth of many tree species. Their beneficial effects are physiological in nature; i.e., increased root absorption surface, selective ion absorption and accumulation, ability to render unavailable substances in soil available to the plant host, etc. (Harley, 1959).

Another possible beneficial role of ectomycorrhizae in tree growth and development has been discussed by Zak (1964). He suggested that ectomycorrhizae may be less susceptible to infection by root pathogens than are nonmycorrhizal short roots; thus, they furnish protection against pathogen invasion. Zak postulated that mycorrhizal fungi may furnish protection by (i) utilizing root carbohydrates and other chemicals which would be attractive to pathogens; (ii) providing a physical barrier to pathogens in the form of the fungus mantle; (iii) secreting antibiotics which inhibit or kill the pathogen; (iv) supporting a protective rhizosphere population of other microorganisms and (v) stimulating the cells of the root during symbiosis to elaborate chemical inhibitors. These inhibitors may not only function to maintain the mycorrhizal fungi in the symbiotic-parasitic relationship, but they may also serve to inhibit infection by pathogens.

The direct involvement of mycorrhizae in protecting roots from infection has been observed by few investigators. Davis, Wright, and Hartley (1942), in an examination of diseases of forest-tree nursery stock, suggested that mycorrhizae may be beneficial by preventing infection of short roots by pseudomycorrhizal fungi and root pathogens. Levisohn (1954) reported that various mycorrhizal pine and spruce seedlings appeared immune to root infection by a *Rhizoctonia*-like fungus. She suggested that these pathogenic infections indicate soil conditions inhibitory to the growth and functioning of a true mycorrhiza former, which normally exercises biological control.

Garrett (1960) describes the rhizosphere as the outermost defense of the plant against attack by root pathogens. This zone of defense normally supports a much greater population of microorganisms than is found in nonrhizospheric soil. Tribunskaya (1955) found approximately ten times as many fungi in rhizospheres of mycorrhizal pine seedlings as in those without mycorrhizae. She concluded that the various fungal symbionts were responsible for the different microflora of the rhizosphere.

Katznelson, Rouatt, and Peterson (1962) showed that mycorrhizal roots of yellow birch (*Betula alleghaniensis* Britton) exerted a stimulatory effect on numbers of certain physiological groups

of soil bacteria and actinomycetes. Bacteria which grew in a simple medium appeared to be suppressed around mycorrhizae, as were total numbers of fungi. Root pathogens, i.e., *Pythium, Fusarium,* and *Cylindrocarpon* spp., predominated in nonmycorrhizal rhizospheres, whereas *Mycelium radicis* (nod. nud.), *Penicillium* spp., and other rapidly growing, nonpathogenic fungi were more prevalent around mycorrhizal roots. *Pythium* and *Fusarium* spp. were absent from the rhizospheres of mycorrhizae.

Neal, Bollen, and Zak (1964) examined the rhizospheres of three morphologically distinct mycorrhizae, adjacent suberized roots, and nonrhizosphere soil from under a single Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) tree. Each microhabitat contained a distinct microflora. These investigators attributed the differences between mycorrhizal rhizospheres to different associated fungus symbionts. They suggested that some mycorrhizae may support a more effective rhizosphere barrier than others and thus affect the degree of infection by root pathogens. Foster and Marks (1967) made electron microscopic examination of *Pinus radiata* D. Don mycorrhizae and found a distinct spatial distribution of bacteria in the rhizosphere. The largest bacterial populations were found in the outermost fungus mantle layers and in areas of the soil colonized by fungus hyphae that could have originated from the mantle. The bacterial population in the outer layers of the mantle was about 16 times that found in the outermost region of the rhizosphere.

The effects of root exudates on root diseases have been investigated in a variety of crop plants. Exudates from certain plants stimulate mycelial growth, germination of microsclerotia, tactic zoospore activity, and virulence of various pathogens. Root exudates from other plants can inhibit the same processes (Rovira, 1965). A limited amount of work has been reported on the chemical nature of exudates of tree roots. Slankis (1958) and Slankis, Runeckles, and Krotkov (1964) found that roots of aseptic white pine (*Pinus strobus* L.) seedlings with needles exposed to $C^{14}O_2$ liberated a complex mixture of more than 35 C^{14} compounds. Their results provided evidence that tree roots, like those of herbaceous plants, liberate many organic metabolites into the rhizosphere.

The process of chemotaxis reportedly is the primary means by which zoospores of phycomycetous fungi initiate infection on host roots. This phenomenon occurs on roots of many plants by zoospores of various *Phytophthora* species (Rovira, 1965), including *P. cinnamomi* Rands (Zentmyer, 1961), *Pythium aphanidermatum* (Edson) Fitzp. (Royle and Hickman, 1963), and *Aphanomyces euteiches* Drechs. (Cunningham and Hagedorn, 1961). In these investigations, the test roots are assumed nonmycorrhizal because the plants were grown under such conditions, i.e., aseptic culture, hydroponics, etc., which would limit mycotrophy. Thus, no reports are available concerning the ability or inability of zoospores to be attracted to mycorrhizal roots of any plant species.

Melin (1963) states that exudates of pine roots are stimulatory to growth of ectomycorrhizal fungi. These exudates contain, besides vitamins and amino acids, one or more growth-promoting substances referred to as M-factors. These substances are not specific to pine roots but are also found in root exudates of other

plants. They are thought to be utilized by the symbiont during both the development and maintenance of mycorrhizae. This suggests that exudates from mycorrhizae present in the rhizosphere may be: (i) those root exudates not utilized by the mycorrhizal symbiont; (ii) chemical by-products from the metabolism of the symbiotic fungus; or (iii) exudates released as the result of the metabolic interaction of the commensal partners. These theoretical changes in the exudates of mycorrhizal roots could alter their attractiveness to certain root pathogens.

Several external and internal morphological barriers are found in plants which mechanically influence either pathogen entrance or its spread in host tissue. Tough outer walls of epidermal cells and suberized root periderm reportedly impede direct penetration by pathogens and may function as mechanical barriers. Other mechanical barriers often are formed after infection. In some plants, suberized healing tissue or cicatricial layers develop, demarcating the localized lesions of infection. The role of these mechanical barriers in the protection of plants in general is not considered to be significant (Burström, 1965).

The fungus mantle of ectomycorrhizae, however, may create a unique and totally different type of mechanical barrier against pathogenic infection. In mature ectomycorrhizae the fungus mantle is a tightly interwoven hyphal network which completely covers the root tip and cortex. This hyphal network usually is complete, i.e., relatively free of voids which expose root tissue to direct contact with the soil and, theoretically, could constitute a mechanical barrier. In order to establish infection in cortex tissue of mycorrhizae, a pathogen must be able to physically or enzymatically penetrate fungus as well as plant cortex tissues. Boosalis (1964), in a review of hyperparasitism, discusses only a few mycoparasites which are also root pathogens. There are reports of fungi nonpathogenic on higher plants which are hyperparasitic on mycorrhizal fungi. An example is *Hypomyces lactifluorum* (Schw. ex Fr.) Tul. reported by Buller (1922) to be parasitic on the hymenium of the sporophore of *Lactarius piperatus* (L. ex Fr.) S. F. Gray, a mycorrhizal symbiont associated with several tree species (Trappe, 1962).

Most plant cells are capable of elaborating inhibitory chemicals during their metabolic response to pathogenic attack. Many of these compounds are inhibitory to pathogens and are considered by many authors to be important in disease resistance (Cruickshank, 1963; Tomiyama, 1963). Plant cells subjected to symbiotic-parasitic invasion have also been reported to respond by the production of substances inhibitory to the fungus symbiont. Bernard (1911) studied the symbiosis between *Rhizoctonia repens* Bernard and orchid tubers, and concluded that an antifungal compound was formed in tubers in response to infection by the fungus symbiont. Subsequent research by Gäumann and Kern (1959), Gäumann (1960), and Gäumann, Nuesch, and Rimpau (1960) has revealed that tubers of several species of *Orchis* and other genera produce orchinol, coumarin, and an unidentified phenolic compound in response to infection by *R. repens*. Orchinol is also produced in tubers of different *Orchis* spp. in response to infection by other species of *Rhizoctonia* and a variety of other endomycorr-

hizal and pathogenic fungi. Orchinol could not be found in uninfected orchid tubers. The inhibitory spectrum of orchinol is wide and not highly specific because it inhibits many fungi and certain bacteria. The production of these inhibitory compounds is considered a defense mechanism of the orchid which keeps the fungus in the symbiotic, rather than the pathogenic, state. Also, the presence of these systemic inhibitory compounds protects the tissue not only against reinfection by the fungus symbiont but also against infection by pathogenic organisms.

Orchids are endomycorrhizae; i.e., the symbiotic infection is intracellular. This is not the case with ectomycorrhizae, where hyphal development in tissue is limited to the Hartig net contained in the middle lamellae. Precisely what keeps the hyphae from penetrating the cellulosic cell walls has not been explained. Melin (1963) proposed that the root produces an inhibiting principle which plays an important part in the establishment and maintenance of symbiotic relationships. The inhibitor may determine the susceptibility of the root to symbiotic infection similar to roots involved in chemotaxis. This inhibitor, combined with a nondiffusible inhibitor present in the cytoplasm as postulated by MacDougal and Dufrenoy (1944), may be the agent controlling the extension of the mycorrhizal fungus hyphae in the root. Foster and Marks (1967) examined ectomycorrhizae of *Pinus radiata* by electron microscopy, and stated that polyphenolic compounds in the tannin layer of the fungus mantle could provide a chemical barrier to soil fungi. These compounds, which possibly limit the ectomycorrhizal fungi to Hartig net parasitism, may also function as inhibitors to root pathogens attempting infection of these mycorrhizal roots.

The production of antibiotics by soil fungi, actinomycetes, and bacteria has been recognized for the past several decades. It was demonstrated by Wright (1956a; 1956b) and others that various saprophytic fungi can produce antibiotics in restricted soil sites, such as pieces of straw and seed coats. It is generally accepted (Brian, 1957; Garrett, 1960; Jackson, 1965) that the resulting antibiotic concentrations are of sufficient quantity to influence significantly the pattern of saprophytic colonization of these sites by microorganisms. However, the significance of antibiotic production by saprophytes in reducing the inoculum potential of root pathogens and of subsequent root disease development is poorly understood.

Most attempts at controlling the activities of root pathogens in soil by soil inoculations with saprophytic organisms which produce antibiotics in the laboratory have failed. An accepted explanation for these failures is that antibiotic production is limited to the immediate substrate or "ecological niche" of the saprophyte (Garrett, 1960). This restricted site of antibiotic production apparently is not of major significance in reducing pathogen inoculum potential outside this site. Theoretically, this need not be the fate of antibiotics produced by mycorrhizal fungi. The "ecological niche" of these specialized root parasites is the host root. The fungi in mycorrhizae are ensured of essential metabolites (e.g., carbohydrates, vitamins, etc.) for which they need exert only minimal competitive efforts. Any antibiotics produced in this "niche" should

be ideally located for inhibitory effects on pathogens attempting infection of these mycorrhizal or perhaps even adjacent nonmycorrhizal roots.

Grand (1964) and Krywolap, Grand, and Casida (1964) reported that *Cenococcum graniforme* (Sow.) Ferd. & Winge, a widespread symbiont on over 100 tree species (Trappe, 1964), produced an antibacterial compound in pure culture, in sclerotia from soil, and while in mycorrhizal relationship with roots of several tree species. The antibiotic was apparently translocated from the mycorrhizae and soilborne sclerotia into roots, stems, and needles of these trees. They speculated that this antibiotic substance could confer some degree of protection to the tree against bacterial invaders.

The production of antibacterial compounds by mycorrhizal fungi in pure culture also has been studied. Morimoto, Iwai, and Fukumoto (1954) reported that the culture filtrate of a *Lactarius* sp., which they found associated with ectomycorrhizae, contained two antibacterial compounds. Santoro and Casida (1959; 1962) presented evidence that extracts of mycelia of *Amanita caesaria* (Scop. ex Fr.) Pers. ex Schw., *A. rubescens* (Pers. ex Fr.) S. F. Gray, *A. muscaria* (L. ex Fr.) Pers. ex Hooker, *Boletus rubellus* Krombh., and *Suillus luteus* (L. ex Fr.) S. F. Gray contained antibacterial compounds. *S. luteus* was the most active of these fungi in the production of a probable polyene antibiotic. Antibacterial compounds have been found in other basidiomycetes which, according to Trappe (1962), are associated with ectomycorrhizae. As discussed by Trappe, this group of mycorrhizal fungi is composed primarily of those fungi which have been linked to probable mycorrhizal associations in nature. Few have been shown definitely to be true symbionts based on controlled inoculations. Accordingly, the following antibiotic producing fungus symbiont relationships are only tentative. Robbins *et al.* (1945) reported that *Paxillus involutus* (Batsch ex Fr.) produces an antibacterial compound in agar plate test. *Clitocybe rivulosa* (Pers. ex Fr.) Kumm. and *Hydnum repandum* L. ex Fr. were reported by Wilkins (1946) to contain antibacterial compounds in their sporophores, and Melin, Wikén, and Öblom (1947) found antibacterial activity in culture filtrates of *Marasmius scorodonius* (Fr.) Fr. Wilkins and Harris (1944) made extracts from sporophores of more than 700 species of higher basidiomycetes and found that over 24 percent contained antibacterial activity. Over 50 of these fungi, mainly *Amanita, Cortinarius, Tricholoma, Lactarius, Hygrophorus,* and *Hebeloma* species can be associated with ectomycorrhizae.

Antifungal activity also has been found in certain mycorrhizal fungi. Rypáček (1960) found that *Suillus variegatus* (Swartz ex Fr.) O. Kuntz. inhibited *Fomes annosus* (Fr.) Cooke and several wood decay fungi. Zak (1964) tested basidiomycetes isolated from slash pine mycorrhizae and found several exhibiting strong antagonism toward *Phytophthora cinnamomi* and *Cylindrocladium scoparium* Morg. Vaartaja and Salisbury (1965) demonstrated that *Suillus granulatus* (L. ex Fr.) O. Kuntz. had a relatively broad spectrum of antifungal activity in agar cultures. Among the fungi inhibited were the root pathogens *Cylindrocarpon radiciola* Wr., *Fusarium solani* (Mart.) App. et Wr. emend Snyder et Han-

sen, *Phytophthora cactorum* (Leb. & Cohn) Schroet., and *Pythium periplocum* Drechs.

Anchel *et al.* (1962) reviewed work on a unique group of polyacetylenic antibiotic substances, called diatretynes, from culture filtrates of several basidiomycetous fungi. Over 14 hymenomycetes produce these antibiotics; of these, *Clitocybe diatreta* (Fr.) Kumm., *C. rivulosa, C. odora* (Bull. ex Fr.) Kumm., and *Lepista nuda* (Bull. ex Fr.) Cooke produced one or more of these compounds and were mycorrhizal associates. The most active of these antibiotics, diatretyne nitrile, is both antifungal and antibacterial. Diatretyne amide, a reduction product of nitrile, is antibacterial, whereas diatretyne 3, a subsequent reduction product of the amide, is weakly antibacterial. These polyacetylene antibiotics are apparently the only ones that have been structurally and chemically identified from cultures of fungi known to be symbionts of ectomycorrhizae.

Šašek and Musílek (1967; 1968) found several mycorrhizal fungi of Scots pine (*Pinus sylvestris* L.) which produced antibiotics effective against gram positive bacteria and selected soil fungi. *Tricholoma saponaceum* (Fr.) Kummer and *Rhizopogon roseolus* (Corda in Sturm) Th. M. Fr. exhibited the greatest antibiotic activity. Based on the foregoing evidence, there is little doubt that ectomycorrhizal fungi as a group have the potential for producing a vast number of different antibiotics. The significance of this production, however, in relation to any root pathogen and root disease development has thus far been only speculation.

The literature reviewed shows that there is circumstantial evidence to support the various mechanisms involved in the root protection concept as proposed by Zak (1964). The purpose of this report is to present the results of research designed to test this concept. The root pathogen used in these tests was *Phytophthora cinnamomi,* the causal organism of littleleaf disease of shortleaf (*Pinus echinata* Mill.) and loblolly (*P. taeda* L.) pines. This fungus pathogen was selected because it: (i) infects the same root tissue invaded by mycorrhizal fungi; (ii) initiates infection by motile zoospores which are reported to be chemically attracted to roots of host plants, thus allowing for tests on the chemical attractiveness of mycorrhizae; and (iii) has hosts such as shortleaf and loblolly pines which are also hosts of several known mycorrhizal fungi.

Materials and Methods

The following techniques and sources of fungi and bacteria have been published in detail (Marx 1969a, 1969b; Marx and Davey 1969a, 1969b) and are only summarized here.

Initial experiments were conducted to study antagonism of mycorrhizal fungi to pathogenic fungi and soil bacteria. The mycorrhizal fungi were grown in several agar and liquid culture media. In agar cultures, the mycorrhizal fungi were grown for 20 days, after which inoculum of the pathogenic fungi was placed at various distances from the colony. Forty-eight different root pathogenic fungi were examined. After incubation, the zones of pathogen growth inhibition were measured. In liquid cultures,

the mycorrhizal fungi were grown for various periods of times at different temperatures. The culture filtrates were sterilized by Millipore filtration and tested to determine their effects on germination of zoospores and vegetative growth of *Phytophthora cinnamomi* and on multiplication of soil bacteria.

In the initial tests, *Leucopaxillus cerealis* var. *piceina* (Peck) ined. produced a very potent antifungal and antibacterial compound in agar and liquid cultures. The following procedures were used to identify the antibiotics and to study the physiology of antibiotic production: The fungus was grown for 50 days at 15°C in liquid medium and the culture filtrates collected. After freeze-drying, the residue was suspended in water adjusted to pH 2.0 and extracted alternately with ethyl acetate and 5 percent sodium bicarbonate. The concentrated extract had high antifungal and antibacterial activity. Ultraviolet and infrared absorption spectra were obtained, and acid hydrolysis of the concentrate was performed to study hydrolytic products of the antibiotics. Physiology of antibiotic production was studied by growing *L. cerealis* var. *piceina* and *Pisolithus tinctorius* (Pers.) Coker & Couch (for comparison) in liquid culture and assaying the culture filtrates at 10-day intervals for antibiotic activity and sequence of antibiotic synthesis. *L. cerealis* var. *piceina* was also grown in root extracts of shortleaf pine, in a substrate of vermiculite and peat moss moistened with nutrients at different acidities, and in supplemented sterile pine humus. These substrates were extracted and assayed for the antibiotics. The influence of diatretyne nitrile at different concentrations on aseptic germination of shortleaf pine seed and seedling growth was also studied.

Mycorrhizae of shortleaf and loblolly pine seedlings were formed in aseptic culture with several different mycorrhizal fungi. The mycorrhizae formed by *L. cerealis* var. *piceina* and the immediate rhizosphere substrate around the mycorrhizae were extracted and assayed for the diatretyne antibiotics. A special root cylinder technique was used to determine relative susceptibility of mycorrhizal and nonmycorrhizal roots of both pine species to infection by *P. cinnamomi*. Individual lateral roots, still intact on pine seedlings and supporting either mycorrhizae formed by the different symbionts or nonmycorrhizal short roots, were inserted through slits in the root cylinders of glass (20 mm diameter by 25 mm deep), sealed, and inoculated with zoospores of *P. cinnamomi*. Observations for chemotaxis of zoospores to the various roots were made microscopically for several hours. After incubation for 3 to 10 days, the roots were examined histologically for pathogenic infection.

Morphologically distinct forms of mycorrhizae of shortleaf pine seedlings developed in greenhouse pot culture in a pine humus-soil mixture and those developed in initially sterile soil mixtures were also inoculated in a similar manner. The susceptibility of cortical cells in mycorrhizae formed by *Thelephora terrestris* (Ehrh.) Fr. that had colonized the sterilized soil mixture in the greenhouse and in nonmycorrhizal short and lateral roots of shortleaf pine seedlings was tested. These were modified by cutting 0.1 or 1 mm from their growing tips, inserting the tips in root cylinders and inoculating them with *P. cinnamomi*. Observations

for chemotaxis of zoospores to the cut surfaces of the roots were made, and after incubation, the roots were examined histologically for infection.

Results

In the antagonism studies, *Laccaria laccata* (Scop. ex Fr.) Berk & Br., *Lactarius deliciosis* (L. ex Fr.) S. F. Gray, *Leucopaxillus cerealis* var. *piceina, Pisolithus tinctorius,* and *Suillus luteus* inhibited growth of nearly half of 48 different fungal root pathogens (table 1). *L. cerealis* var. *piceina* inhibited 92 percent of the test pathogens. Culture filtrates of this fungus symbiont were strongly inhibitory to *Phytophthora cinnamomi* and soil bacteria. Zoospore germination was inhibited completely in filtrates of this symbiont. Maximum antibiotic production occurred during the rapid growth phase in liquid culture. Length of culture incubation and temperature strongly influenced production of antibiotics by *L. cerealis* var. *piceina.* It grew best from 10-20 C, whereas *Pisolithus tinctorius* grew best from 30-35°C in liquid culture.

Based on biological and spectrophotometric analyses of the filtrates of *L. cerealis* var. *piceina,* the antibiotics were identified as diatretynes. Maximum production of diatretyne nitrile, which is strongly antifungal and antibacterial, occurred during the rapid and incipient autolysis growth phases in liquid medium. During continued autolysis, the nitrile was reduced to diatretyne amide and diatretyne 3, which are antibacterial only.

Table 1.—*Antagonism of five ectomycorrhizal fungi to a variety of root pathogenic fungi in agar medium* [1]

Test Pathogen	*Laccaria laccata*	*Lactarius deliciosus*	*Leucopaxillus cerealis* var. *piceina*	*Pisolithus tinctorius*	*Suillus luteus*
Armillaria mellea	–	–	–	–	–
Cylindrocladium scoparium	–	–	+	–	+
Fomes annosus	–	–	+	–	–
Fusarium oxysporum var. *pini*	–	–	–	–	+
Phytophthora spp. (9) [2]	+11% [3] –89%	+83% –17%	+100% –0%	+0% –100%	+100% –0%
Polyporus tomentosus var. *circinatus*	–	+	+	–	–
Poria weirii	–	–	+	–	+
Pythium spp. (24)	+67% –33%	+0% –100%	+100% –0%	+0% –100%	+92% –8%
Rhizoctonia spp. (8)	+0% –100%	+13% –87%	+75% –25%	+0% –100%	+25% –75%
Sclerotium bataticola	+	–	+	–	–
Pathogens Inhibited [4]	35%	16%	92%	0%	76%

[1] Antagonism is expressed as either "+" indicating mycelial growth of the pathogen was inhibted or "–" indicating the pathogen was not inhibited.

[2] The number in parentheses following a genus denotes the number of species tested.

[3] The percentage value is based on the number of species of the various genera of pathogens which were (+) or were not (–) inhibited.

[4] Overall % pathogen inhibition by all mycorrhizal fungi was approximately 44% (101 of 231).

The structures of the diatretyne antibiotics follow:

$HOOC\text{-}CH=CH\text{-}C\equiv C\text{-}C\equiv C\text{-}C\equiv N$ Diatretyne nitrile

$HOOC\text{-}CH=CH\text{-}C\equiv C\text{-}C\equiv C\text{-}CONH_2$ Diatretyne amide

$HOOC\text{-}CH=CH\text{-}C\equiv C\text{-}C\equiv C\text{-}C\equiv C\text{-}CH_2OH$ Diatretyne 3

Concentrations of diatretyne nitrile causing minimum inhibition (20 percent) to germination of zoospores of *Phytophthora cinnamomi* were 50 to 70 ppb with total (100 percent) inhibition at 2 ppm. Approximately half of the inhibited zoospores germinated upon removal (dialysis) of diatretyne nitrile between these concentrations, indicating a fungistatic effect. Zoospores failed to germinate after removal of higher concentrations, indicating these levels were lethal. Concentrations that caused minimum inhibition to vegetative growth of *P. cinnamomi* were detected at 0.5 ppm, with total inhibition at 9 ppm. Soil bacteria were inhibited between 0.5 and 2.5 ppm. *L. cerealis* var. *piceina* produced approximately 12 ppm of diatretyne nitrile in liquid culture after 50 days growth at 15°C. Diatretynes were also produced by this fungus in an extract of shortleaf pine roots, in substrates of pH 4.0 to 6.6, and in sterile pine humus supplemented with sucrose or malt extract. Diatretyne nitrile did not inhibit germination of aseptic shortleaf pine seeds exposed for 1 or 2 hours to concentrations up to 40 ppm, but it was phytotoxic to aseptic shortleaf seedlings at concentrations exceeding 10 ppm. *L. cerealis* var. *piceina* also produced diatretyne nitrile and diatretyne 3 in mycorrhizae and rhizospheres of shortleaf pine seedlings in aseptic culture.

In the inoculation tests of aseptic seedlings, only 25 percent of nonmycorrhizal short roots of shortleaf pine seedlings adjacent to mycorrhizae formed by *L. cerealis* var. *piceina* were infected by zoospores of *P. cinnamomi*, whereas 100 percent on nonmycorrhizal short roots of control seedlings and short roots adjacent to mycorrhizae formed by *Pisolithus tinctorius* and by *L. laccata* were infected. Only 77 percent of the short roots of shortleaf pine and 85 percent of those of loblolly pine adjacent to mycorrhizae formed by *S. luteus* were infected (tables 2 and 3). Fully developed

Table 2.—Phytophthora cinnamomi *infection of roots of aseptic shortleaf pine seedlings with and without mycorrhizae*

	Inoculation with zoospores of *Phytophthora cinnamomi*						
		Mycorrhizae		Short roots		Lateral root tips	
Mycorrhizal fungus	Number of seedlings	Number inoculated	Percent infected	Number inoculated	Percent infected	Number inoculated	Percent infected
Laccaria laccata	9	7	0	29	100	8	100
Leucopaxillus cerealis var. *piceina*	6	27	0	32	25	6	100
Pisolithus tinctorius	9	42	19 [1]	13	100	6	100
Suillus luteus	8	9	0	34	77	6	100
Nonmycorrhizal control	7	....	....	23	100	8	100

[1] Mycorrhizae with incomplete mantle and Hartig net development.

Table 3.—Phytophthora cinnamomi *infection of roots of aseptic loblolly pine seedlings with and without mycorrhizae*

Mycorrhizal fungus	Inoculation with zoospores of *Phytophthora cinnamomi*						
		Mycorrhizae		Short roots		Lateral root tips	
	Number seedlings	Number inoculated	Percent infected	Number inoculated	Percent infected	Number inoculated	Percent infected
Laccaria laccata	8	8	0	9	100	4	100
Pisolithus tinctorius	9	29	21 [1]	16	100	4	100
Suillus luteus	7	12	17 [1]	47	85	6	100
Non-mycorrhizal controls	7	....	....	36	100	8	100

[1] Mycorrhizae with incomplete mantle and Hartig net development.

mycorrhizae formed by *L. laccata, L. cerealis* var. *piceina,* and *S. luteus* on shortleaf pine were resistant to *Phytophthora cinnamomi.* Mature mycorrhizae formed on loblolly pine by *L. laccata* were also resistant. Tests on mycorrhizal synthesis with *L. cerealis* var. *piceina* on loblolly pine were incomplete. Incomplete mantle development on the root meristem by *S. luteus* on loblolly and by *Pisolithus tinctorius* on both pines, permitted infection, but *Phytophthora cinnamomi* did not spread into the Hartig net region of the mycorrhizae. Short-root initials covered by fungus mantles from adjacent mycorrhizae were also resistant to infection, whereas those not covered by mantles were highly susceptible. Chemotaxis of zoospores to mycorrhizal and nonmycorrhizal roots of both pines in this series of tests was not observed.

Ectomycorrhizae formed on shortleaf pine seedlings in greenhouse pot culture of nonsterile pine humus were also resistant to *P. cinnamomi* (table 4). Although mature mycorrhizae were resistant to infection, some mycorrhizae with incomplete fungus man-

Table 4.—Phytophthora cinnamomi *infection of roots of shortleaf pine seedlings grown in non-sterile humus in greenhouse pot culture*

Root type	Infection by *Phytophthora cinnamomi*			
	3 days after inoculation		10 days after inoculation	
	Number inoculated	Percent infected	Number inoculated	Percent infected
Mycorrhizal form 1	12	25 [1]	14	43 [1]
Mycorrhizal form 2	17	0	7	0
Mycorrhizal form 3	23	0	11	0
Non-mycorrhizal short root	16	100	11	100
Non-mycorrhizal lateral root tip	6	100	6	100

[1] Mycorrhizae with incomplete fungus mantle and Hartig net development.

tles at the root meristem were infected, but the pathogen did not penetrate the Hartig net region.

Mycorrhizae formed in initially sterile soil by *Thelephora terrestris* with artificially exposed cortex cells with Hartig nets were resistant to infection, although stelar tissues of these mycorrhizae were susceptible when exposed directly to *P. cinnamomi* (table 5). Zoospores were attracted to exposed stelar tissues of mycorrhizae, nonmycorrhizal short roots, and lateral root tips.

Table 5.—Phytophthora cinnamomi *infection of detached and modified roots of shortleaf pine seedlings grown in autoclaved humus in greenhouse pot cultures*

Items	Infection of indicated root type						
	Mycorrhizae			Short roots		Lateral root tips	
	Complete	Cortex exposed	Stele exposed	Complete	Tips removed	Complete	Tips removed
Number Inoculated	7	9	11	5	13	5	5
Percent Infected	0	0	100 [1]	100 [2]	100 [2]	100 [2]	100 [2]

[1] Stelar tissue infected only.
[2] Cortex and stelar tissues infected.

Discussion

The basic hypothesis set forth by Zak (1964) and others that ectomycorrhizae are resistant to pathogenic attack and are protective barriers against infection is valid, based on the results of this research. Therefore, another benefit to the health of higher plants is derived from the ectomycorrhizal state, the benefit of built-in biological control against infections of feeder roots by pathogens.

The mechanical barrier reportedly created by fungus mantles of mycorrhizae apparently does function as some type of barrier to root pathogenic infection. Short root initials covered only by mantles extending from adjacent mycorrhizae and mycorrhizae with cortex cells free of Hartig net development but with mantle coverings were devoid of infection by *Phytophthora cinnamomi*. Short root initials and cortex cells of nonmycorrhizal roots of control seedlings were infected heavily by intracellular hyphae of the pathogen in all instances.

The proposed mechanism concerning the role of antibiotics produced by mycorrhizal fungi in reducing susceptibility of mycorrhizae to infection could not be determined absolutely. The possible antibiotic influence was obscured because the presence of fungus mantles on mycorrhizae was sufficient to limit infection. There were indications, however, that production of diatretyne nitrile by *Leucopaxillus cerealis* var. *piceina* in mycorrhizae was a factor in reducing susceptibility of adjacent nonmycorrhizal short roots. Only 25 percent of the short roots of shortleaf seedlings grown with this symbiont were infected, compared to 100 percent infection of short roots of most other seedlings with mycorrhizae and

all of the control seedlings. It was not determined whether the antibiotic was translocated to these roots from the mycorrhizae or simply absorbed by them from a site of production in the rhizosphere. It is also possible that the mycorrhizae absorbed the antibiotic from the rhizosphere. Unfortunately, nonmycorrhizal short roots adjacent to these mycorrhizae were not tested for the presence of diatretyne compounds. Reduction of susceptibility of short roots adjacent to mycorrhizae formed by *Suillus luteus* also was evident. This symbiont was only weakly antagonistic to *P. cinnamomi,* but it did demonstrate strong antagonism to closely related fungus pathogens. Precisely why certain short roots adjacent to these mycorrhizae were not infected by *P. cinnamomi* must await further research efforts. Future research also will be necessary to assess the value of antibiotic production by the various pine mycorrhizal fungi in respect to infection of mycorrhizae and short roots by the various other root pathogens. Most of these other pathogens, including species of *Pythium* and *Phytophthora* which were very sensitive to these antibiotics, have not been tested for pathogenicity on pine roots.

Cortex cells enclosed by a Hartig net and exposed to *P. cinnamomi* remained free of infection. This indicates that the Hartig net could be a mechanical barrier to infection or that nondiffusable inhibitors were present as postulated by MacDougal and Dufrenoy (1944) and Foster and Marks (1967). Direct evidence was not found to support either of these mechanisms.

Although differences in chemotaxis of zoospores of *P. cinnamomi* were not detected between mycorrhizal and nonmycorrhizal roots of shortleaf or loblolly pines, differences were found in germination of zoospores and growth of germ tubes on roots. At regions of cell elongation on short and lateral roots, zoospores germinated rapidly and germ tube elongation was quite vigorous. This was not evident on mycorrhizal roots because zoospore germination and germ tube growth processes were similar to those on suberized lateral root segments. This indicates, although only indirectly, that the mycorrhizae were not as chemically attractive to the zoospores as were nonmycorrhizal roots.

In most tests, the involvement of antagonistic rhizosphere populations was removed, especially in those tests involving aseptically formed mycorrhizae. Populations of rhizosphere bacteria may have been established on these roots from bacteria contained in the nonsterile zoospore inoculum. This, however, was considered to be of minimal significance because zoospores of *P. cinnamomi* in most instances settled on the roots and germinated in a few hours. The naturally occurring roots on seedlings grown in pots may have had a high population of rhizosphere microorganisms, but the root-washing procedure should have reduced this population to a low level. Therefore, it was concluded that the proposed root protection mechanism of antagonistic rhizosphere organisms was not responsible for the resistance of mycorrhizal roots to infection in these tests. This is not to suggest, however, that this mechanism is not operative under more natural conditions.

Most of Zak's (1964) proposed mechanisms of root protection were probably functional, at one time or another, in these tests.

Several of the mechanisms, such as presence of the mantle and Hartig net barriers, antibiotic production, and nondiffusable inhibitors, are very difficult to separate in that they occur simultaneously. This, of course, insures a wider range of protection by mycorrhizae because rarely could one mechanism function exclusively.

The general conclusion is that ectomycorrhizae of shortleaf and loblolly pines are protective barriers to pathogenic infection by *P. cinnamomi.* In field conditions, one could expect trees with abundant development of mycorrhizae to have less susceptible root tissue exposed to *P. cinnamomi,* and perhaps other feeder root pathogens, than trees with few or no mycorrhizae.

We have recently found evidence in the field to support this conclusion: A single morphological type of ectomycorrhizae, discovered by Powell, Hendrix and Marx (1968), became dominant on roots of pecan (*Carya illinoensis* (Wangenh.) K. Koch) following soil application of nematocides and fungicides for control of necrosis of feeder roots. Sporophores of *Scleroderma bovista* Fr. were found abundantly in this orchard shortly after soil treatments. Mycelial strands from sporophores were traced directly to mycorrhizae. The various soil treatments did not significantly reduce populations of either parasitic nematodes or *Pythium* species, pathogens associated with necrosis of feeder roots of pecan (Hendrix and Powell, 1968), but symptoms of root damage gradually disappeared. A few months after treatment, the trees had dense crowns, dark green leaves, and only a few necrotic roots. Untreated trees had thin crowns, chlorotic leaves, necrotic roots, and few mycorrhizae. The mycorrhizae of the untreated trees were not morphologically similar to those on the treated trees. There appeared to be a correlation between increase in vigor of the treated pecan trees and increase in the development of a single ectomycorrhizal type. Isolates of *S. bovista* obtained from both sporophores and mycorrhizae were culturally identical (Marx and Bryan, 1969). These isolates formed mycorrhizae on roots of pecan seedlings in the greenhouse which were morphologically similar to those observed in the field. The most significant finding, however, was the antibiotic production by isolates of *S. bovista.* The fungus produced a very potent antibiotic in agar culture which effectively inhibited growth of various *Pythium* species. The most sensitive of these fungi were *Pythium spinosum* Sarvada, *P. irregulare* Buisman, and *P. vexans* deBary, which are also the pathogens most consistently associated with necrosis of pecan feeder roots. It appeared that the various soil treatments eliminated microbial competitors which enhanced the development of mycorrhizae by *S. bovista.* Stimulation of tree growth following soil treatment probably resulted from mycorrhizal effects such as (i) greater absorbing capacity of root systems having large numbers of mycorrhizae and (ii) antagonism of *S. bovista* to root pathogens. This sensitivity of the root pathogens to antibiotics produced by *S. bovista,* the only apparent fungus symbiont on the pecan roots, adds further evidence to the possible role of ectomycorrhizae as biological deterrents to infection of roots by pathogens.

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Separate No. FS-288

8.

Effect of Nematodes on Root-Inhabiting Fungi

Jerry W. Riffle

Several nematode species that occur in tree rhizospheres are principally mycophagous in nature, and many of these nematodes have been found in forest nurseries. Species of *Aphelenchoides*, *Aphelenchus*, and *Ditylenchus* were found in at least 16 of 35 forest nurseries in eight southeastern states (Hopper, 1958). Two of these genera, *Aphelenchus* and *Aphelenchoides*, were common in three forest nurseries in Quebec (Sutherland, 1965), whereas *Ditylenchus* and *Aphelenchoides* species were associated with coniferous seedlings in most of 91 national forest nurseries surveyed in eastern Japan (Mamiya, 1969).

Mycophagous nematodes also occur in natural forest stands. An *Aphelenchoides* sp. was commonly found in soil samples from a forested area in the Bartlett Experimental Forest in New Hampshire (Shigo and Yelenosky, 1960). Another *Aphelenchoides* sp. (now *A. cibolensis*) was one of the most widely distributed nematodes found in the rhizosphere of conifers in low elevation *Pinus ponderosa* Laws. stands in central New Mexico (Riffle, 1968). *Aphelenchoides* spp. have been found associated with 28 forest tree species, while *Ditylenchus* spp. were associated with 23 forest trees (Ruehle, 1967).

Little information is available on the effects of mycophagous nematodes on root-inhabiting fungi, especially the mycorrhizal fungi. The objectives of this paper are to present (1) a review of the literature about the effects of mycophagous nematodes on root-inhabiting fungi and (2) the results of investigations of the effects of a mycophagous nematode on the growth of root-pathogenic and mycorrhizal fungi.

Review of the Literature

Effect of Mycophagous Nematodes on Nonmycorrhizal Root-Inhabiting Fungi

Cytoplasmic streaming is frequently inhibited in hyphae parasitized by nematodes. Such streaming was halted a short time after the stylet of either *Ditylenchus myceliophagus* J. B. Goodey or *D. destructor* Thorne penetrated a hyphal cell of *Botrytis cinerea* (Doncaster, 1966). By the injection of digestive juices, *D. destructor* was able to abruptly terminate the cytoplasmic streaming of *Chaetomium indicum* Corda (Anderson, 1964).

In addition to stopping cytoplasmic streaming, nematode feeding alters hyphal cytoplasm. As *Paurodontoides linfordi* (Hechler) Jairajpun and Siddiqi fed on hyphal cell contents of nine test fungi, the cytoplasm became more granular and less vacuolate (Hechler, 1962). At no time during the feeding process were granules ob-

served flowing from the stylet tip into the hyphal cell. When *Ditylenchus destructor* injected digestive juices into hyphal cells of *Chaetomium indicum,* the cytoplasm immediately became more translucent and more uniform in density than in normal hyphal cells (Anderson, 1964). A dome-shaped zone usually appeared around the tip of the stylets of *D. myceliophagus* and *D. destructor* when they fed on large hyphal cells of *Botrytis cinerea* (Doncaster, 1966). The zone was bounded by granules that became increasingly conspicuous, but the granulation in other cells seemed to follow paths across the cell or to become general.

Shrinkage of hyphal cells may occur as a result of nematode feeding. Such shrinkage was conspicuous when *D. destructor* fed on *C. indicum, Monascus* sp., and *Penicillium megasporum* Berk. and Br. (Anderson, 1964). The shrunken cells did not appear to recover, and any hyphal branch buds that formed prior to feeding did not develop (Anderson, 1964). Sometimes *B. cinerea* cells penetrated by *D. myceliophagus* or *D. destructor,* and up to five consecutive cells adjoining them, shrank within four hours, and their contents lost all recognizable structure in less than 12 hours (Doncaster, 1966).

After mycophagous nematodes withdraw their stylets from hyphal cells, cellular contents frequently gush out in considerable quantities through the stylet punctures. Cytoplasm has been observed flowing from such punctures after *D. destructor* and *D. myceliophagus* fed on *B. cinerea* (Doncaster, 1966), after *Aphelenchus avenae* fed on *Pythium arrhenomanes* (Rhoades and Linford, 1959), and after *D. destructor* fed on *Oidiodendron nigrum* Robak (Anderson, 1964). A continuous series of bubbles was formed along the cell length of *O. nigrum* immediately after the cellular contents escaped.

Effects of nematode feeding in fungal hyphae are not necessarily limited to penetrated cells. Modification of hyphal cytoplasm induced by *Paurodontoides linfordi* on several fungi, progressed from cell to cell during the feeding period, often as far as six cells away from the stylet (Hechler, 1962). Similar effects on cells adjacent to penetrated cells have been observed on *Chaetomium indicum* (Anderson, 1964) and *Botrytis cinerea* (Doncaster, 1966). Hechler (1962) suggested that nematodes injected a large amount of an extra-oral digestive enzyme during feeding, and this enzyme affected the cells adjacent to the stylet. She observed a sudden shrinkage of the dorsal duct of *P. linfordi* at the beginning of intestinal contraction. Since this nematode feeds for a long period of time at one site, she speculated that the nematode obtained food from three to six cells on either side of the stylet.

Hyphal cells adjacent to cells penetrated by the stylet of a nematode can be killed as the result of a single feeding. After a cell of *Chaetomium indicum* damaged by *Ditylenchus destructor* died, an adjacent cell usually died 30 minutes later, and the next cell did not die for another two hours. As many as five cells died from the effects of a single feeding, but in no case did cells die after 16 hours (Anderson, 1964).

Mycophagous nematodes can reduce or even stop the growth of fungi. *Ditylenchus destructor* fed on 64 fungi and reduced the growth of many of the isolates (Faulkner and Darling, 1961).

Aphelenchus avenae reduced and even stopped the growth of some phytopathogenic fungi (Mankau and Mankau, 1963). This nematode species also stopped the growth of *Pythium arrhenomanes* when they fed several times at or near hyphal tips (Rhoades and Linford, 1959).

Mycophagous nematodes can cause death of their fungal hosts. *Aphelenchus avenae* killed five species of nematode trapping fungi when both were grown in dual cultures for 30 days (Cooke and Pramer, 1968). This nematode essentially destroyed the mycelium of *Rhizoctonia solani, Fusarium solani* f. *pisi,* and *F. solani* f. *phaseoli* in sand cultures (Klink and Barker, 1968). *Armillaria mellea* and *Verticillium albo-atrum* also can be destroyed by *A. avenae* (Mankau and Mankau, 1963). These investigators found that fungi from cultures in which nematodes had reached large populations often failed to revive when transferred to new dishes. They indicated that when new growth occurred it was probably from thick-walled chlamydospores, conidia, or other survival structures which *A. avenae* did not destroy.

Some mycophagous nematodes feed on and even kill conidia and other survival structures of fungi. As *Eudorylaimus ettersbergensis* (de Man) Andrássy inserted its stylet into conidia of a *Cephalothecium* sp., the conidia instantly lost their turgor and collapsed like balloons (Hollis, 1957). Similarly, the conidia of *Verticillium albo-atrum* collapsed when drained of their contents by *Aphelenchoides besseyi* (Nickle and McIntosh, 1968). Sclerotia of *Rhizoctonia solani* and *Sclerotium* sp. produced numerous new sclerotia when added to moistened silica sand, but when 100 *Aphelenchus avenae* were added at the same time, sclerotia did not form (Klink and Barker, 1968). The nematodes destroyed the mycelium growing out of the initial sclerotium and prevented the formation of new sclerotia.

Table 1 lists mycophagous nematode parasites of some common root-inhabiting fungi and references for each.

Effect of Nematodes on Mycorrhizal Fungi

Few investigations have been made on the effects of mycophagous nematodes on the growth of mycorrhizal fungi. Linear growth of *Suillus granulatus* (L. ex Fr.) Kuntze was greatly reduced by *Aphelenchoides cibolensis* (Riffle, 1967). The nematode reproduced readily on cultures of this fungus, and many cultures failed to revive when transferred to a fresh medium. *Aphelenchus avenae* reduced the diameter growth of *Amanita rubescens* (Fr.) S. F. Gray, *Cenococcum graniforme* (Sow.) Ferd. & Winge, *Rhizopogon roseolus* (Cda.) Th. Fr., *Russula emetica* (Fr.) S. F. Gray, *S. granulatus, S. luteus* (Fr.) S. F. Gray, and *S. punctipes* (Peck) Sing. after 28 days of feeding (Sutherland and Fortin, 1968). None of the fungi were killed, even though removal of hyphal cytoplasm caused the hyphal walls to collapse or hyphal tips to shrivel for some distance on each side of the stylet.

Effect of Nematodes on Ectomycorrhizae of Conifers

Pinus echinata Mill. mycorrhizae parasitized by *Criconemoides rusticum* (Micoletzky) Taylor became excessively spongy and swollen, and the mantle and Hartig net were absent (Jackson,

Table 1.—*Root-inhabiting fungi reported as hosts of mycophagous nematodes—with references*

Fungus host	Mycophagous nematode	References
Alternaria solani (Ell. & G. Mart.) L. R. Jones & Grout	*Aphelenchus avenae* Bastian	Pillai & Taylor, 1968
	Aphelenchus avenae	Townshend, 1964
	Bursaphelenchus fungivorus Franklin & Hooper	Townshend, 1964
	Ditylenchus destructor Thorne	Faulkner & Darling, 1961
	Ditylenchus sp. (bisexual)	Pillai & Taylor, 1968
	Ditylenchus sp. (parthenogenetic)	Pillai & Taylor, 1968
	Paurodontoides linfordi	Pillai & Taylor, 1968
	Paraphelenchus acontioides Taylor & Pillai	Pillai & Taylor, 1968
Armillaria mellea (Fr.) Quél	*Ditylenchus destructor*	Faulkner & Darling, 1961
Botrytis cinerea Pers. ex Fr.	*Aphelenchoides limberi* Steiner	Hooper, 1962
	Aphelenchus avenae	Townshend, 1964
	Bursaphelenchus fungivorus	Franklin & Hooper, 1962; Townshend, 1964
Fusarium culmorum (W. G. Smith) Sacc.	*Aphelenchus avenae*	Townshend, 1964
	Bursaphelenchus fungivorus	Townshend, 1964
Fusarium oxysporum Schlchtendahl	*Paurodontoides linfordi*	Hechler, 1962
Fusarium oxysporum f. *lycopersici* (Sacc.) Snyder & Hansen	*Aphelenchus avenae*	Townshend, 1964
	Bursaphelenchus fungivorus	Townshend, 1964
	Ditylenchus destructor	Faulkner & Darling, 1961
Fusarium oxysporum f. *niveum* (E. F. Sm.) Snyder & Hansen	*Ditylenchus destructor*	Faulkner & Darling, 1961
Fusarium oxysporum f. *pisi* (Linford) Snyder & Hansen	*Aphelenchus avenae*	Townshend, 1964
	Bursaphelenchus fungivorus	Townshend, 1964
Fusarium solani (Mart.) Appel & Wr.	*Aphelenchus avenae*	Mankau & Mankau, 1963; Pillai & Taylor, 1968; Townshend, 1964
	Aphelenchoides parietinus (Bastian) Steiner	Katznelson & Henderson, 1964
	Bursaphelenchus fungivorus	Townshend, 1964
	Ditylenchus sp. (bisexual)	Pillai & Taylor, 1968
	Ditylenchus sp. (parthenogenetic)	Pillai & Taylor, 1968
	Paurodontoides linfordi	Pillai & Taylor, 1968
	Paraphelenchus acontioides	Pillai & Taylor, 1968

Table 1.—*Root-inhabiting fungi reported as hosts of mycophagous nematodes—with references*—Continued

Fungus host	Mycophagous nematode	References
Fusarium solani f. *phaseoli* Burk.	*Aphelenchoides besseyi* Christie	Nickle & McIntosh, 1968
	Aphelenchoides sacchari Hooper	Nickle & McIntosh, 1968
	Aphelenchus avenae	Nickle & McIntosh, 1968
	Paurodontoides linfordi	Nickle & McIntosh, 1968
Fusarium solani var. *pisi* (F. R. Jones) Snyder & Hansen	*Ditylenchus destructor*	Faulkner & Darling, 1961
Gibberella zeae (Schw.) Petch	*Ditylenchus destructor*	Faulkner & Darling, 1961
Helminthosporium sativum Pammel, King, & Bakke	*Aphelenchoides parietinus*	Katznelson & Henderson, 1964
	Paurodontoides linfordi	Hechler, 1962
Leptographium sp.	*Aphelenchoides parietinus*	Katznelson & Henderson, 1964
Ophiobolus graminis Sacc.	*Aphelenchus avenae*	Townshend, 1964
	Bursaphelenchus fungivorus	Townshend, 1964
Phoma lingam (Fr.) Desm.	*Ditylenchus destructor*	Faulkner & Darling, 1961
Phytophthora cactorum	*Ditylenchus* sp. (bisexual)	Pillai & Taylor, 1968
	Ditylenchus sp. (parthenogenetic)	Pillai & Taylor, 1968
Pythium arrhenomanes Drechsler	*Aphelenchus avenae*	Rhoades & Linford, 1959
	Paurodontoides linfordi	Hechler, 1962
Pythium debaryanum Hesse	*Aphelenchus avenae*	Chin & Estey, 1966; Townshend, 1964
	Bursaphelenchus fungivorus	Townshend, 1964
Pythium irregulare Buis.	*Paurodontoides linfordi*	Pillai & Taylor, 1968
	Paraphelenchus acontioides	Pillai & Taylor, 1968
Pythium ultimum Trow	*Aphelenchus avenae*	Townshend, 1964
	Bursaphelenchus fungivorus	Townshend, 1964
Rhizoctonia solani Kühn	*Aphelenchoides besseyi*	Nickle & McIntosh, 1968
	Aphelenchoides parietinus	Katznelson & Henderson, 1964
	Aphelenchoides sacchari	Nickle & McIntosh, 1968
	Aphelenchus avenae	Chin & Estey, 1966; Mankau & Mankau, 1963; Nickle & McIntosh, 1968; Pillai & Taylor, 1968; Townshend, 1964
	Bursaphelenchus fungivorus	Townshend, 1964
	Ditylenchus sp. (bisexual)	Pillai & Taylor, 1968
	Ditylenchus sp. (parthenogenetic)	Pillai & Taylor, 1968

Table 1.—*Root-inhabiting fungi reported as hosts of mycophagous nematodes—with references*—Continued

Fungus host	Mycophagous nematode	References
Rhizoctonia solani Kühn	*Paurodontoides linfordi*	Pillai & Taylor, 1968; Nickle & McIntosh, 1968
	Paraphelenchus acontioides	Pillai & Taylor, 1968
Sclerotium bataticola Taubenhaus	*Aphelenchus avenae*	Chin & Estey, 1966
	Paurodontoides linfordi	Hechler, 1962
Thielaviopsis basicola (Berk.) Ferr	*Aphelenchus avenae*	Chin & Estey, 1966; Mankau & Mankau, 1963
Trichoderma viride (Pers.) Fr.	*Aphelenchus avenae*	Townshend, 1964
	Bursaphelenchus fungivorus	Townshend, 1964
	Ditylenchus destructor	Baker et al., 1954; Faulkner & Darling, 1961
Verticillium albo-atrum Reinke & Berth	*Aphelenchoides besseyi*	Nickle & McIntosh, 1968
	Aphelenchoides parietinus	Katznelson & Henderson, 1964
	Aphelenchoides sacchari	Nickle & McIntosh, 1968
	Aphelenchus avenae	Chin & Estey, 1966; Nickle & McIntosh, 1968; Townshend, 1964
	Bursaphelenchus fungivorus	Townshend, 1964
	Ditylenchus destructor	Faulkner & Darling, 1961
	Paurodontoides linfordi	Nickle & McIntosh, 1968

1948). Swollen females of a *Heterodera* sp. were found embedded in *P. monticola* Dougl. rootlets and they appeared destructive to the mycorrhizae (Nickle, 1960). Similar *Meloidogyne* females caused hypertrophy of *Pinus ponderosa* Laws. mycorrhizal rootlets (Riffle and Lucht, 1966), and *M. javanica* (Treub) Chitwood was found in close association with swollen mycorrhizae of *P. elliottii* Engelm. (Donaldson, 1967). Zak (1967) found an undescribed *Meloidodera* sp. on two of six morphologically distinct *Pseudotsuga menziesii* (Mirb.) Franco mycorrhizae. Mature females were abundant on a pale olive, irregularly pinnate form, but only occasional on a white, ramiform mycorrhiza.

Hoplolaimus tylenchiformis Daday and *Meloidodera floridensis* Chitwood, Hannon, and Esser penetrated both lateral and mycorrhizal short roots of *Pinus elliottii* and *P. taeda* L. (Ruehle, 1962). *Hoplolaimus tylenchiformis* migrated in and fed on cortical tissue of the rootlets and caused extensive internal damage. Giant cells developed in undifferentiated tissue in the meristematic region in tips of mycorrhizae infected with *M. floridensis.*

Difficulty in establishing rooted *Rhododendron* cuttings was traced to a mycophagous *Deladenus* sp. associated with a deficiency of mycorrhizae (Clark, 1964). Microscopic examination of the

roots revealed very little external mycelium, but well-developed mycorrhizae were seen on roots of healthy plants. Clark suggested that the nematode fed on the mycorrhizae and destroyed the external mycelium as rapidly as it developed.

In pure culture, the abundance of *Suillus granulatus* mycorrhizae of *Pinus resinosa* Ait. was significantly reduced five weeks after inoculation with *Aphelenchus avenae* (Sutherland and Fortin, 1968). The authors suggested that a population in excess of 10,000 nematodes per 500 ml flask was necessary before reduction occurred. No nematodes were found upon or within the mycorrhizae, and they did not alter the morphology of the mycorrhizae.

This work is summarized in table 2.

Table 2.—*Plant-parasitic nematodes known to parasitize mycorrhizal short roots of conifers*

Nematode species	Conifer	Reference
Criconemoides rusticum	*Pinus echinata*	Jackson 1948
Hoplolaimus tylenchiformis	*Pinus taeda*	Ruehle 1962
	Pinus elliottii	Ruehle 1962
Heterodera sp.	*Pinus monticola*	Nickle 1960
Meloidogyne javanica	*Pinus elliottii*	Donaldson 1967
Meloidogyne sp. (undescribed)	*Pinus ponderosa*	Riffle and Lucht 1966
Meloidodera sp. (undescribed)	*Pseudotsuga menziesii*	Zak 1967
Meloidodera floridensis	*Pinus taeda*	Ruehle 1962
	Pinus elliottii	Ruehle 1962

Current Studies

Aphelenchoides cibolensis Riffle, was isolated from *Pinus ponderosa* mycorrhizal rootlets in 1965. The following experiments were made to determine the effects of this nematode on root-pathogenic and mycorrhizal fungi.

Effects of Aphelenchoides cibolensis *Riffle on Root-Pathogenic Fungi*

Materials and Methods

An experiment was made to determine if *A. cibolensis* could feed on, reproduce on, and affect the growth rate of five root-pathogenic fungi. The fungi tested were *Fomes annosus* (Fr.) Cke., *Polyporus schweinitzii* Fr., *Leptographium* sp., *Poria weirii* Murr., and *Armillaria mellea*. Populations of the nematode were increased and maintained on several cultures of *Suillus granulatus* growing on potato dextrose agar (PDA).

Sixteen 1-cm discs were cut from 3-week-old cultures of each fungus and placed on malt agar in the center of individual 90-mm petri dishes. After 48 hours, a suspension of 192 $\pm$ 6(24)[1] aseptic nematodes was added to eight colonies of each fungus. The remaining eight colonies were not inoculated and served as controls. Linear diameter growth of the colonies was measured to the

[1] These numbers refer to the mean plus or minus the standard error of the mean, with the number of observations in parentheses. Other similar notations will appear throughout the text.

nearest mm immediately after addition of the nematodes and at 5-day intervals thereafter for a period of 35 days. During this time temperatures ranged from 20 to 23°C.

The experiment was terminated 35 days after the nematodes were added. Small squares of medium containing mycelium were cut from the margin of each nematode-inoculated colony and transferred to fresh malt agar to determine if the fungi would revive. All of these cultures were incubated at 21 to 23° C for at least one month before the final readings were taken.

Each nematode-inoculated fungal colony was placed in a modified Baermann funnel to liberate and condense the nematodes for population counts. After 24 hours, the nematodes were drawn off and populations determined by counting several aliquots from each sample.

Results

Armillaria mellea was killed by nematode feeding (table 3). Its linear growth was essentially stopped after 25 days of feeding. The first reduction in aerial growth of this fungus was observed after 15 days of feeding, and by 25 days, all aerial growth in all cultures was completely consumed. In addition, rhizomorphs formed in two of the eight control dishes, but no rhizomorphs were formed by the fungus in plates that contained nematodes.

Nematodes increased very readily on the *Leptographium* species, but they failed to reduce its linear growth or viability (table 3). This fungus grew rapidly and completely covered the 90-mm petri dish in 10 days. The nematodes reproduced rapidly on this abundant supply of mycelium. First reduction in aerial mycelium was apparent in 20 days and in 25 days it was consumed. One possible reason why this fungus remained viable was that it produced a

Table 3.—*Effect of 35 days of feeding by* Aphelenchoides cibolensis *on the mean linear growth and viability of five root-pathogenic fungi, and ratio of final to initial nematode populations on these fungi*

Root-pathogenic fungus	Linear growth of inoculated fungi as percent of controls [1]	Percent inoculated fungi killed [2]	Population ratio [3]
Armillaria mellea (OKM 2911S) [4]	67	100	1060
Poria weirii (OKM 4742SP)	85	0	733
Leptographium sp. (ASC 343)	100	0	2460
Polyporus schweinitzii (OKM 4435S)	100	0	0
Fomes annosus (PCL 251–301)	100	0	0

[1] Mean diameter growth of 8 colonies for each fungus.

[2] Percent of 8 colonies that failed to revive when transferred to a fresh medium after the experiment.

[3] Number of times final population increased over an initial population of 192 ± 6(24) nematodes.

[4] Letters and numbers following fungus names refer to culture source: OKM —Dr. O. K. Miller; ASC—Albuquerque Stock Culture; and PCL—Dr. P. C. Lightle.

large number of conidia, which probably germinated and produced colonies when transferred to the fresh medium. The nematode was not observed feeding on conidia.

The linear growth of *Poria weirii* was reduced by nematode feeding, but there was no effect on its viability (table 3). First reduction in aerial growth occurred in 20 days, and at the end of the experiment, only 13 percent remained.

The nematode had no effect on the growth or viability of *Polyporus schweinitzii* or *Fomes annosus* (table 3). These fungi, like the *Leptographium* species, grew rapidly and covered the petri dish in 10 days. The nematode failed to maintain a population on these fungi, and it is possible that they produced a metabolite that was toxic to the nematode or inhibited its reproduction. Bassett (1967) found that extracts of culture filtrates of *F. annosus* were toxic to *Pinus taeda* seedlings, *Chlorella pyrenoidosa* Check, six bacterial species, and mice.

Effect of Aphelenchoides cibolensis *Riffle on Mycorrhizal Fungi*

Material and Methods

Fifty-three fungi were tested with *Aphelenchoides cibolensis* in two separate experiments. Twenty-four of these fungi were isolated from sporocarps collected from mixed conifer stands in central New Mexico:

Agaricus arvensis Schaeff. ex Fr. (JR810)[2]
Amanita muscaria (L. ex Fr.) Pers. ex Hooker (JR775)
Amanita pantherina (D. C. ex Fr.) Schum (JR847)
Amanitopsis sp. (JR807)
Boletus edulis Bull. ex Fr. (JR839)
Cantharellus cibarius Fr. (JR740)
Clitocybe sp. (JR806)
Clitocybe sp. (JR691)
Collybia dyrophila (Bull. ex Fr.) Kummer (JR829)
Cortinarius sp. (HB1256)[3]
Flammulina velutipes (Curt. ex Fr.) Sing. (JR606)
Hygrophorus chrysodon (Batsch ex Fr.) Fr. (JR818)
Hygrophorus purpurascens (Fr.) Fr. (JR816)
Lactarius deliciosus (L. ex Fr.) S. F. Gray (JR803)
Lactarius uvidus (Fr. ex Fr.) Fr. (JR696)
Leccinum subrobustum Smith, Thiers, & Watling (JR667)
Leucopaxillus amarus f. *bicolor* (Murr.) Singer & Smith (JR682)
Pholiota squarrosa (Pers. ex Fr.) Kummer (JR700)
Rhizopogon sp. (JR848)
Russula sp. (JR722)
Suillus granulatus (JR674)
Suillus lakei (Murr.) Sing. (JR824)
Tricholoma sp. (JR837)
Xerocomus truncatus Singer, Snell, & Dick (JR836)

The remaining 29 fungi were obtained from research scientists of the USDA Forest Service.

[2] Numbers in parentheses following fungus names represent collection numbers.

[3] Dr. Hal Burdsall collection number 1256.

In the first experiment, ten 5-mm discs of each of 38 fungi were cut from 1-month-old cultures. Discs of 28 fungi were placed on PDA in the center of individual 90-mm petri dishes, while discs of the remaining 10 fungi were placed on similar agar at one end of individual Ryan growth tubes. After 48 hours, a suspension of $201 \pm 11(40)$ aseptic nematodes was added to five colonies of each fungus. The remaining five colonies were not inoculated and served as controls.

The experiment was continued for 40 days; linear growth of the colonies was measured to the nearest millimeter every four days. Temperature ranged from 20 to 23° C during the experiment.

Techniques for determining fungal viability (capability to revive when transferred to a fresh medium) and final nematode populations on each fungus were the same as those described in the previous study with root-pathogenic fungi.

In the second experiment, procedures and materials were identical to those used in the first except that (1) only 15 fungi were used and these were replicated eight times, (2) 90-mm petri dishes were used, (3) the original inoculum level was $241 \pm 17(25)$, and (4) the experiment was terminated 28 days after the nematodes were added.

Results

First Experiment: Reduction in linear growth and aerial mycelium of most fungi was first apparent after nematodes had fed 8 to 12 days. After 40 days of feeding, the nematodes had little effect on the linear growth of 19 fungi; their growth was 75 to 100 percent of their respective controls. Growth of 12 other fungi was reduced to 51 to 73 percent, and growth of the remaining 7 was reduced to 50 percent or less of their controls. *Hypholoma fasciculare* is an example of a fungus whose linear growth was little affected by the nematode. Its aerial mycelium, however, was completely consumed after 24 days of feeding. A fungus whose linear growth was severely affected was *Leucopaxillus amarus* f. *bicolor*. Its mean linear growth was stopped 16 days after the nematodes were added, and after 40 days, its mean colony diameter was only one-fourth that of its controls.

Nematodes concentrated at the periphery of the fungus colonies and fed on hyphal cells. Upon contacting a hyphal cell of *Suillus granulatus*, the nematode pressed its lips firmly against the cell wall. Its stylet and neck were at right angles to the longitudinal axis of the cell as it started to penetrate the cell (figure). The stylet was thrust vigorously until the wall was penetrated, and then it was inserted at least one-half the distance into the hyphal cell. No injection of salivary secretions was observed. The median bulb began pulsating shortly after the stylet was inserted. The nematode fed on *S. granulatus* cells for one or two minutes. During feeding, cytoplasm was observed flowing toward the stylet orifice. The median bulb valve pulsated very rapidly at the end of each feeding, and bubbles usually appeared in the hyphal cell at the feeding site.

The viability of 15 fungi was not affected by nematode feeding, but 11 failed to revive when transferred to a fresh medium.

Aphelenchoides cibolensis reproduced readily on many of the test fungi. Their numbers increased over 100 times the initial popu-

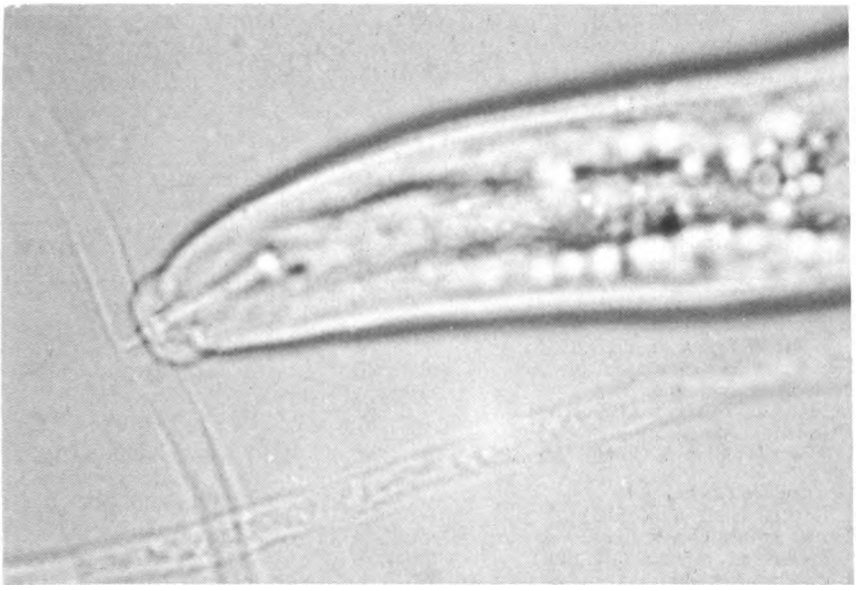

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Figure.—*Aphelenchoides cibolensis* feeding on a *Suillus granulatus* hypha.

lation on more than half of the fungi. On *Clitopilus prunulus,* the population increased from 201 to nearly 473,000 nematodes.

Nematodes failed to maintain a population on *Pleurotus corticatus, Paxillus panuoides,* and *Pholiota squarrosa.* In addition, they were not able to maintain a population in dishes that contained an agar medium but lacked a fungus. The nematodes remained active for 20 to 28 days, surviving on body food reserves, but then their activity gradually declined until they appeared moribund on the agar surface. Eggs were not observed in any of these dishes (table 4).

Second Experiment: Effects of *Aphelenchoides cibolensis* on linear growth and viability of 15 additional known or suspected mycorrhizal fungi were similar to those obtained in the first experiment. The nematode had little effect on the linear growth of seven fungi, but the growth of three was reduced to 50 percent or less of their controls. Five fungi failed to revive when transferred to a fresh medium. The aerial mycelium of these fungi was consumed in 21 to 28 days. The viability of six other fungi was not affected. Two of these fungi, *Tricholoma* sp. and *Clitocybe* sp., grew slowly and supported a small nematode population. Perhaps these fungi produced a substance that was toxic to the nematodes, for abundant aerial mycelium still remained after 28 days of feeding. The nematodes increased over 100 fold on three of the fungi, but failed to increase over 10 fold on five others (table 5).

Discussion

Aphelenchoides cibolensis fed and reproduced on 53 of the 58 fungi used in this investigation. If a host is defined as "a source from which a nematode can obtain sufficient food to sustain growth

Table 4.—*Effect of 40 days of feeding by* Aphelenchoides cibolensis *on the mean linear growth and viability of 38 known or suspected mycorrhizal fungi, and ratio of final to initial nematode populations on these fungi*

Mycorrhizal fungus	Growth of inoculated fungi as percent of controls [1]	Percent inoculated fungi killed [2]	Population ratio [3]
		Petri plates	
Hygrophorus piceae Kühn & Romagn. (BZ S115) [4]	22	100	45
Leucopaxillus amarus f. *bicolor*	25	20	35
Lactarius aurantiacus (Fr.) S. F. Gray (BZ S109)	42	40	508
Pisolithus tinctorius (Pers.) Coker & Couch (BZ S31)	43	100	265
Crinipellis campanella (Peck) Sing. (BZ S15)	50	100	335
Truncocolumella citrina Zeller var. *citrina* (BZ S28)	51	100	70
Phallus ravenelii Berk. and Curt. (BZ S26)	52	100	18
Cenococcum graniforme (EH28)	60	100	169
Suillus granulatus	62	40	646
Russula emetica (EH4)	67	20	1662
Inocybe lacera (Fr.) Kumm. (BZ S120)	68	80	75
Leccinum subrobustum	68	100	19
Corticium bicolor Peck. (BZ S12)	71	100	578
Suillus ponderosus Smith & Thiers (BZ S105)	73	100	143
Amanita muscaria (BZ S–80)	77	100	636
Calvatia subcretacea Zeller (BZ S52)	82	80	1349
Clitopilus prunulus (Scop. ex Fr.) Kumm. (BZ S7)	82	0	2353
Suillus borealis Smith, Thiers, and Miller (BZ S99)	85	100	285
Russula sp.	87	0	60
Lepista personata (Fr. ex Fr.) Cooke (BZ S29)	90	80	1948
Hypholoma fasciculare (Huds. ex Fr.) Korst (BZ A3)	91	20	13
Cantharellus floccosus Schw. (BZ S10)	97	0	45
Alpovah cinnamoneus Dodge (BZ S128)	97	20	9
Pleurotus corticatus (Fr. ex Fr.) Quél (DM)	98	0	0
Leucopaxillus albissima (Peck) Singer var. *albissima* (DM)	98	0	110
Paxillus panuoides (Fr. ex Fr.) Fr.	99	0	0
Flammulina velutipes	100	0	216
Thelephora terrestris (Ehr.) Fr. (BZ S67)	100	20	23
		Ryan growth tubes	
Polyporus montanus (Quél) Ferry (BZ S42)	47	0	21
Rhizopogon roseolus (EH83)	48	0	291
Amanita rubescens (EH8)	51	20	164
Pholiota squarrosa	60	0	0

Table 4.—*Effect of 40 days of feeding by* Aphelenchoides cibolensis *on the mean linear growth and viability of 38 known or suspected mycorrihizal fungi, and ratio of final to initial nematode populations on these fungi*—Continued

Mycorrhizal fungus	Growth of inoculated fungi as percent of controls [1]	Percent inoculated fungi killed [2]	Population ratio [3]
		Petri plates	
Macrolepiota procera (Scop. ex Fr.) Sing (DM)	71	0	634
Suillus variegatus (Swartz ex Fr.) O. Kuntze (BZ S14)	89	20	1
Panus rudis Fr. (DM)	92	0	357
Cantharellus cibarius	97	0	58
Lactarius uvidus	97	0	518
Clitocybe sp. (JR691)	100	0	1

[1] Mean growth of five colonies, diameter growth in plates, and radial growth in tubes per fungus.

[2] Percent of five colonies that failed to grow when transferred to a fresh medium after the experiment.

[3] Number of times final population increased over initial population of 201 ± 11(40) nematodes.

[4] Letters and numbers following fungus names refer to culture source: BZ—Dr. B. Zak; EH—Dr. E. Hacskaylo; DM—Dr. D. H. Marx; and JR—Dr. J. W. Riffle.

Table 5.—*Effect of 28 days feeding by* Aphelenchoides cibolensis *on the mean linear growth and viability of 15 known or suspected mycorrhizal fungi, and ratio of final to initial nematode populations on these fungi*

Mycorrhizal fungus	Growth of inoculated fungi as percent of controls [1]		Percent inoculated fungi killed [2]	Population ratio [3]
	substrate	aerial		
Amanitopsis sp.	17	0	100	27
Boletus edulis	45	0	100	26
Collybia dryophila	50	31	0	19
Rhizopogon sp.	55	4	38	48
Amanita pantherina	59	0	100	37
Amanita muscaria (JR775)	65	0	100	112
Agaricus arvensis	65	65	50	4
Suillus lakei	75	75	16	10
Cortinarius sp.	76	0	0	116
Clitocybe sp. (JR806)	77	55	0	5
Hygrophorus purpurascens	85	0	100	15
Lactarius deliciosus	89	16	0	93
Tricholoma sp.	90	79	0	6
Hygrophorus chrysodon	90	0	38	1
Xerocomus truncatus	97	0	0	932

[1] Mean diameter growth of eight colonies per fungus.

[2] Percent of eight colonies that failed to grow when transferred to a fresh medium after the experiment.

[3] Number of times the population increased over an initial population of 241 ± 17(25) nematodes.

and multiplication", then these 53 fungi could be classed as hosts of this nematode. Good hosts are characteried by high nematode populations. Twenty-six of the 53 fungi are considered good hosts because *A. cibolensis* reproduced readily on them and increased over 100 times their initial population levels.

During active fungal growth, nematodes concentrated in large numbers around the advancing margin of the colonies. After growth was halted and aerial mycelium was consumed, nematodes were distributed over the entire agar surface. Perhaps the nematodes reached a population ceiling in these cultures and further increase was limited by lack of food. In many cultures, large masses of nematodes migrated up the glass of the petri dishes and swarmed in macrosopic clusters. Such behavior is probably caused by a lack of food or the accumulation of metabolic products in the cultures.

Aphelenchoides cibolensis had little effect on the linear growth of 31 of the 58 fungi used in this investigation. Many of these fungi grew rapidly on the medium used. The linear growth of 10 others, however, was reduced to 50 percent or less of their controls and several of these grew slowly. It has been shown that when fungal growth is restricted by nutritional or environmental factors, the effects of *Aphelenchus avenae* on fungal growth can be severe (Cooke and Pramer, 1968). Perhaps the 10 fungi severely affected by *A. cibolensis* did not obtain sufficient nutrition from the PDA medium to support vigorous growth. This possibility deserves further investigation.

The viability of 25 fungi was not affected by *Aphelenchoides cibolensis,* but 16 mycorrhizal fungi and 1 root-rot fungus failed to revive when transferred to fresh medium at termination of the experiments.

The following five categories illustrate the effects of the nematode on the growth of many of the fungi used in this investigation:

Group I: Fungi usually grew rapidly on PDA, and little or no reduction in linear growth or viability occurred. For these fungi, the rate of mycelial formation was greater than the rate at which it was being destroyed by the relatively low initial nematode population. The nematodes reproduced rapidly on the abundant food supply and a large nematode population developed. This population consumed all aerial mycelium. Examles of fungi in this group are: *Clitopilus prunulus, Cortinarius* sp., *Flammulina velutipes, Lactarius uvidus, Leptographium* sp., *Leucopaxillus albissima* var. *albissima, Macrolepiota procera, Panus rudis, Poria weirii, Rhizopogon roseolus,* and *Xerocomus truncatus.*

Group II: Fungi usually showed little or no reduction in linear growth or fungus viability, and small nematode populations developed. These fungi might have produced metabolic products that killed the nematodes or inhibited their feeding or reproduction. Examples of fungi in this group are: *Agaricus arvensis, Cantharellus cibarius, C. floccosus, Clitocybe* sp., *Fomes annosus, Paxillus panuoides, Pholiota squarrosa, Pleurotus corticatus, Polyporus schweinitzii, Russula* sp., *Suillus variegatus,* and *Tricholoma* sp.

Group III: Fungi had reduced linear growth and viability, and large nematode populations developed. Examples of fungi in this

group are: *Amanita rubescens, Calvatia subcretacea, Lepista personata, Russula emetica,* and *Suillus granulatus.*

Group IV: Fungi failed to revive when transferred to a fresh medium. Linear growth was reduced and aerial mycelium was completely destroyed. These fungi were usually slow growers and supported low nematode opulations. Examples of fungi in this group are: *Amanita pantherina, Amanitopsis* sp. *Boletus edulis, Hygrophorus piceae, Phallus ravenelii, Leccinum subrobustum,* and *Truncocolumella citrina* var. *citrina.*

Group V: Fungi were similar to those of group IV, except they supported large nematode populations. Examples of fungi in this group are: *Amanita muscaria, Armillaria mellea, Cenococcum graniforme, Corticium bicolor, Crinipellis campanella, Pisolithus tinctorius, Suillus borealis,* and *S. ponderosus.*

This investigation has shown that a mycophagous nematode can feed on, reduce the growth of, and even cause the death of some mycorrhizal fungi under laboratory conditions. Although these conditions are highly artificial, they indicate that mycophagous nematodes have the potential to reduce or inhibit the formation of mycorrhizae under natural conditions. This concept is not new, for mycophagous nematodes have suppressed the development of fungi on the roots of several plants (Klink and Barker, 1968; Rhoades and Linford, 1959; Schindler and Stewart, 1956).

The rhizospheric environment offers many opportunities for nematode-fungus interrelationships. These complex interrelations need to be understood before the role of such organisms in the etiology of root disorders can be fully appreciated.

Acknowledgments

The author extends his thanks and appreciation to Drs. John Palmer and Orsen K. Miller, Forest Disease Laboratory, Laurel, Md.; to Dr. Edward Hacskaylo, Forest Physiology Laboratory, Beltsville, Md.; to Dr. Donald Marx, Southeastern Forest Experiment Station, Athens, Ga.; and to Drs. James Trappe and Bratislav Zak, Pacific Northwest Forest and Range Experiment Station, Corvallis, Oreg., for supplying some of the cultures used in this investigation. Dr. Harold Burdsall, Forest Disease Laboratory, Laurel, Md., and Drs. Trappe and Miller identified some of the fungi used in the study, and their help is greatly appreciated.

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Separate No. FS–289

9.

Nonpathogenic Organisms Associated with Mycorrhizae

C. B. Davey

That portion of the edaphon which involves the biological gradient from soil to root has long been called the rhizosphere (Hiltner, 1904). This region has been further subdivided by Clark (1949), Rawlings (1958), and Foster and Marks (1967). Clark separated the root surface, which he called rhizoplane, from the remainder of the rhizosphere and thus defined both a two dimensional and a three dimensional habitat. More recently, Rawlings and Foster and Marks recognized nonmycorrhizal roots as having a rhizosphere and mycorrhizal roots as having a mycorrhizosphere. Since most plants are mycorrhizal this term is rarely needed, but, it does define the system as it actually is. However, this rather lengthy word should not be further prefixed by ecto- or endo- or any other wholly or partially descriptive adjectives, regardless of their etymological appeal.

Characteristics of Root Zone

The volume of the mycorrhizosphere has not been critically analyzed. Its boundaries are not spherical but rather, resemble a misshapen cylinder whose radius varies with the genotype and age of the plant, the physical and chemical properties of the soil, and the kinds of microorganisms involved. Most people will agree that when the R/S (rhizosphere/soil) ratio (or should it now be called the M/S ratio?) for any organism or group of organisms reaches unity, the soil proper has been reached. In one study of 3-week-old lupines the microflora in general reached this boundary at 18 mm (Papavizas and Davey, 1961). This boundary was pronounced for highly rhizophilic organisms, which were abundant only on the rhizoplane, and nonexistant for those rhizophobic organisms, which always had fractional R/S ratios. In a study of bacteria *in situ* in the mycorrhizosphere, the outer edge was apparently reached 16μ from the "tannin layer" (Foster and Marks, 1967). The bacteria were described as forming a shell in and about the mantle with populations dropping abruptly on both sides.

The mycorrhizosphere is a very complex habitat in which a large number of organisms are acting on and reacting to each other and to the host plant. It is characterized by the presence of: (1) Many exudates of the roots and mycorrhizal fungi. (2) Sloughed root cells and lysed hyphal cells. (3) Various nutrients existing in a range of forms; valances and oxidation states and chelated, sorbed on cation exchange sites, or free. (4) An atmosphere which ranges from highly aerobic to anaerobic and contains varying but relatively high contents of CO_2 and occasionally CO, NH_3, CH_4, H_2S, HCN, and a range of other volatile, low molecular weight metab-

olites. (5) Acidity (pH) which varies in intensity, capacity, and physiological effect depending on its origin from H or Al ions, the cation exchange capacity, and its percent base saturation. (6) Moisture, which is usually under tension and moves under conditions of unsaturated flow. This moisture is variously enriched by exudates and nutrients which have been solublized through the weathering action of the microflora, and it is depleted by the uptake of nutrients and metabolites by roots via diffusion and root or mycorrhiza interception.

Since other discussions in the proceedings are devoted specifically to the relation of mycorrhizae to pathogens (including nematodes), nitrogen fixation, and weathering of minerals, we shall limit our discussion of those subjects to calling attention to their importance and mentioning them only when related to other topics.

Establishment of Mycorrhizae

The earliest relationship possible between mycorrhizae and rhizosphere organisms occurs during entry and establishment of the mycorrhizal fungus in the host root. Although numerous monoxenic mycorrhizal associations have been demonstrated, certain suspect fungi have consistently failed to produce mycorrhizae in pure culture. These recalcitrant fungi may have failed to infect in pure culture because they must act in conjunction with other organisms. Mosse (1962) found that sterile germinated *Endogone* spores never formed mycorrhizae with sterile seedlings. Two pseudomonads were encountered in non-sterile cultures which allowed mycorrhiza formation. These bacteria could be added before, with, or following the *Endogone* with equal success. Other bacteria with pectolytic activity were then tried and found to be effective. No bacteria were ever found within the mycorrhizal roots.

Shemakhanova (1962) reported that formation of pine mycorrhizae was best supported by the presence of *Trichoderma lignorum, Azotobacter chroococcum,* and fluorescent bacteria in the rhizosphere. Malyshkin (1955) also reported that *Azotobacter* and *Trichoderma* stimulated mycorrhiza formation. Vedenyapina (1955) ascribed the value of *Azotobacter* in mycorrhiza formation not to either pectolytic activity or nitrogen fixation but, rather, to the production of thiamin which stimulated both the mycorrhizal fungi and the plant roots. There is no doubt that certain mycorrhizal fungi require thiamin, but whether it comes from rhizosphere microbes or the host plant itself needs further study. West (1939) reported thiamin to be a regular component of sterile root exudates of flax.

Undoubtedly the most elaborate mechanism yet envisaged as operative in mycorrhiza formation was described by Voznyakovskaya and Ryzhkova (1955). They found no protopectinase in pure cultures of several mycorrhizal fungi. In ecological studies, they found abundant nitrogen-fixing, pectinolytic, and cellulolytic bacteria and *Trichoderma* associated with the mycorrhizae. Their conclusion was that the nitrogen-fixing bacteria provide nitrogen for the pectinolytic and cellulolytic bacteria and ligninolytic *Trichoderma.* These four organisms working in consort separate the root cortex cells so that the mycorrhizal fungus can enter. This all rep-

resents quite a coordinated team effort. Although it might well happen, it doesn't seem as though the mycorrhizal condition would be as ubiquitous as it is if any such elaborate arrangement of organisms in time and space was generally required. It does suggest, however, that more attention to the use of dixenic or even polyxenic cultures in mycorrhizal studies is needed. This is true not only in studies of mycorrhiza establishment but also, or even more so, in studies of mycorrhiza function.

Before turning our attention to mycorrhiza function, we should note that there is evidence that rhizosphere organisms may also have detrimental effects on mycorrhiza formation. Brian *et al.* (1945) established that the failure of *Boletus bovinus* to form mycorrhizae was attributable to gliotoxin produced by certain penicillia. Antagonism by bacteria (Sideri and Zolotun, 1952), actinomycetes (Shemakhanova, 1962), and other fungi (Levisohn, 1957) has also been reported.

Stimulation of the development of mycorrhizae by indole compounds has been indicated by Hacskaylo (1957), Slankis (1958), and Moser (1959). *Pseudomonas* spp. often produce indoleacetic acid in the plant rhizosphere (Alexander, 1961) and thus could stimulate mycorrhizal development. Cook and Lochhead (1959) reported that amino acids found in the rhizosphere originated with the host but that growth promoting substances (vitamins and hormones) were products of the microflora.

Organisms Associated with Established Mycorrhizae

The usually observed stimulation of plant growth which is associated with the mycorrhizal condition may well be primarily attributed to the mycorrhizae, although the possibility of a pronounced effect caused by an altered root or rhizosphere population must also be considered. *Fusarium* has often been suggested as being a beneficial, root-invading fungus despite its reputation as a pathogen. In three separate studies, species of *Fusarium* were found to range from beneficial to harmful (Bilai, 1955; Dorokhova, 1955; Khrushcheva, 1955). Khrushcheva and Dorokhova both observed that the beneficial fusaria were restricted to the root cortex while the harmful forms penetrated the stele. Bilai reported that the fusaria were beneficial unless the plant was weakened. This has been observed in soybeans growing in fumigated soil which was inoculated with various rhizoplane organisms. *Fusarium* was stimulatory to plant growth unless nematodes were also introduced. Then the *Fusarium* plots contained plants which were more stunted than those which received only the nematodes (Ross and Davey, unpublished [1]).

Danielson (1966) isolated fungi from surface-sterilized mycorrhizae of loblolly pine (*Pinus taeda* L.) grown in fumigated nursery soil. He was able to recognize three distinct forms of mycorrhizae. *Fusarium* spp. regularly were found with only one of the three types. This type also contained two species of *Chaetominum*. Chesters and Parkinson (1959) studied the succession of rhizosphere fungi on maturing roots of oats and found the fusaria about mid-way in the succession.

[1] Ross, J. R. and C. B. Davey. North Carolina State University, Raleigh, N.C., unpublished data.

Trichoderma must be mentioned because of its ubiquitous occurrence in soil. Its relationship to mycorrhiza, however, may be often indirect. In many studies, it has been found to be mildly or strongly rhizophobic, being very restricted on the rhizoplane (Thomas and Parkinson, 1967) and apparently not common within plant roots. Danielson (1966) recovered *Trichoderma* from less than 2 percent of the pine root segments grown in non-fumigated soil. Yatazawa *et al.* (1960), in soil treated with allyl alcohol, found very high numbers of *Trichoderma* in *Pinus radiata* rhizosphere. The seedlings were exceptionally large and succulent. Dorokhova (1955) found several non-mycorrhizal organisms in wheat root cortex which were digested by the host and hence probably beneficial.

Algae have been reported intimately associated with plant roots, apparently beneath the zone of light penetration. Shtina *et al.* (1964) studied algae in the rhizosphere of several crops. They reported that the number of algae found varied with plant species and age. Young plants and trees did not have high numbers of rhizosphere algae. Green algae were associated with sugar beets, blue-green algae and some diatoms, with cotton, and all types of algae, with rice. They inoculated corn soil with all types of algae but only in the rhizosphere did they find numbers above that of the control. In an inoculation study with blue-green algae to determine N-fixation, they found *Azotobacter* populations rose about 5 times and *Clostridium* about 6 times in the soil. They concluded that certain algae may either fix nitrogen or precondition the environment for nitrogen fixation by bacteria.

Danielson (1966) surprisingly isolated 15 algae from surface sterilized mycorrhizal loblolly pine roots. They were identified as species in the genera *Myrmecia* and *Gloeocystis*. Cytological sections revealed the presence of the algae within root cortex cells. The root sectioning was done after the roots had been incubated and the algae detected visually. Consequently, there remains some question as to the time of cortical cell penetration. However, Wittmann *et al.* (1965) reported blue-green algae to be internal to cycad roots. In Danielson's study, the algae were recovered from only 1.3% of the mycorrhizal roots cultured. This may be significant, however, since only 5% of the same roots yielded possible mycorrhizal fungi.

Another somewhat more remote connection between algae and mycorrhizae was reported by Melin (1959) but attributed to Leibundgut. In this case, the presence of certain lichens on the forest floor reduced mycorrhiza formation.

Many of the conclusions reached by various workers are based on dilution plate techniques. One problem with this method is its inability to determine whether colonies arose from spores or active cells. Agnihothrudu (1955) attempted to make the distinction in samples taken from both the soil and the rhizospheres of several plants. In the soil, 70 to 90% of the fungi existed as spores, while in the rhizospheres of several crops, 55 to 98% were vegetative. During plant flowering, the percent vegetative rose to 83 to 98. The only exception was *Brassica* which had a low percentage of active fungi in the rhizosphere and which has been reported as being one of the few plants which may not form mycorrhizae.

Methods described in the two recent papers of Ramachandra Reddy (1968a, 1968b) might be quite useful in studying reactions of active root and rhizosphere organisms. His technique was to spray the foilage with a material and, in effect, treat the root and rhizosphere from the inside out. In an earlier study, Davey and Papavizas (1961) demonstrated that foliar-applied streptomycin did not alter the number of rhizosphere bacteria but drastically changed the proportion of gram positive to gram negative bacteria in the rhizosphere. The effect lasted up to 12 days.

Only a very few studies have been designed to investigate the specificity of selection of organisms accompanying mycorrhizae. Oswald and Ferchau (1968) isolated and identified bacteria from coniferous roots. They found 51 species of which 7 were only on nonmycorrhizal roots, 22 were only on mycorrhizal roots, and 22 were common to both. A very interesting observation was recorded by O'hara and Hamada (1967) concerning the fairy ring mycorrhizal fungus, *Tricholoma matsutake* (S. Ito et Imai) Singer. No bacteria or actinomycetes were found directly beneath the ring of fruiting bodies. Ahead of the expanding ring they were abundant. In the soil through which the ring had passed, very few bacteria or actinomycetes were found.

Katznelson and co-workers (1956) conducted numerous studies on the rhizosphere organisms as affected by soil conditions and host plant. For example, in one study they characterized the bacterial flora of the rhizosphere. Then they allowed the plant to nearly wilt. When the soil was remoistened they found increased amounts of amino acids in the root exudates. The number of amino acid requiring bacteria in the rhizosphere markedly increased.

One study was specfically designed to investigate the microflora associated with mycorrhizal and nonmycorrhizal roots of yellow birch (*Betula alleghaniensis)* (Katznelson *et al.*, 1962). Both types of roots were isolated and then either washed and plated or washed, homogenized, and plated. Seven types of bacteria were counted and found to be from one to three orders of magnitude more abundant on mycorrhizal roots than on nonmycorrhizal roots. Likewise, actinomycetes were one order of magnitude more abundant with mycorrhiazl roots. Nonmycorrhizal roots yielded species of 8 genera of fungi including 3 pathogen-containing genera. Forty percent of the nonmycorrhizal root segments yielded no fungi at all, while all mycorrhizal roots yielded fungi. They included species of 14 genera, of which only one contains pathogens. The actual fungal counts were reduced in the mycorrhizal roots, but the authors suggested that this indicated simply that the number of spores was reduced while the active hyphae were increased. They stated that the lack of *Penicillium, Aspergillus,* and *Trichoderma* from nonmycorrhizal roots probably indicated the effectiveness of the washing technique in removing spores. They suggested that many of the hyphae of the outer mantle may be lysed and thus be responsible for the release of quantities of metabolites. Finally they indicated that rapid ammonification with mycorrhizal roots could be quite important in forest humus. In a more recent study, Neal *et al.* (1964) found that the fungal symbiont in Douglas-fir mycorrhizae alters the rhizosphere microflora.

Fontana and Luppi (1966) studied the saprophytic fungi associ-

ated with mycorrhizae of pine, larch, oak, chestnut, and corylus. Fungi from 66 taxa were isolated. Phycomycetes were not found, but ascomycetes, hyphomycetes, and sterile dark forms were abundant. They found that the fungal population was generally more abundant and richer with mycorrhizal than with nonmycorrhizal roots; the specific composition being determined by all the factors which influence the rhizosphere, by the mycorrhizal fungus, and by the type of mantle.

Finally, we can cite at least two types of animals which are associated with mycorrhizae. Sutherland and Fortin (1968) demonstrated that the mycophagous nematode *Aphelenchus avenae* may consume the mantle of ectomycorrhizae. Also, some small mites (*Acarina*) appear to be found fairly regularly associated with mycorrhizal roots (Danielson, 1966). They do have a direct effect even if their occurrance is by chance in that their fecal pellets, which are very quickly colonized by the mycorrhizal fungi, presumably provide a readily available source of nutrients.

Conclusion

The works cited in this resumé are typical of many studies which have indicated stimulation of plant growth from various rhizosphere, rhizoplane, and root cortex-penetrating organisms. Levisohn (1957) noted that many plants may benefit from their rhizosphere flora before they become mycorrhizal. Over 20 years ago, Harley (1948) stated that nonmycorrhizal organisms may stimulate plant growth and suggested that mycorrhizal studies should be done in conjunction with rhizosphere studies. With but a few notable exceptions, this suggestion has not been heeded. It is still a very sound suggestion.

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10.

Mycorrhizae and Nutrient Mobilization

G. K. Voigt

In its fundamental form plant growth is a process in which energy and chemical elements are combined. For higher plants solar radiation is the energy source, and the chemical elements involved are ultimately of geologic or atmospheric origin. Entry of these elements into the biosphere invariably involves some facet of microbial activity, usually in combination with higher plants. Mycorrhizae are a specific example of this relationship and, for at least 75 years, they have been identified with nutrient absorption. The purpose of this paper is to examine the possible role that the mycorrhizal association may play in nutrient cycle initiation.

The concept of nutrient cycling in forest ecosystems is now well established and many studies have been carried out to characterize the components involved (Riekirk and Gessel, 1965; Bormann and Likens, 1967; Switzer *et al.*, 1968). Distribution of forest vegetation generally coincides with sufficient precipitation to produce leaching, hence nutrient loss occurs. Ecosystem progression is maintained by specific kinds of input that vary with the nutrient involved. For those elements originating in the geologic substrate, ions may be released by many kinds of reactions (Reiche, 1950; Keller, 1957), but it is likely that biological weathering induced by soil microorganisms is dominant. It has been demonstrated that both bacteria and fungi release ions from minerals (Duff *et al.*, 1963; Katznelson *et al.*, 1962a; Henderson and Duff, 1963; Schatz, 1963; Webley *et al.*, 1963; Jacks, 1965; Voigt, 1965a; Barber, 1968). Biochemical degradation of primary minerals probably involves metabolic CO_2 which generates H ions in the soil system (Keller and Frederickson, 1952; Voigt 1962). Of equal or greater importance are metabolic by-products which have been shown to dissolve primary mineral components (Schatz *et al.*, 1954, 1957; Duff and Webley, 1959; Schatz, 1963; Boyle, *et al.*, 1967). Thus, nutrient ions of geologic origin are mobilized for incorporation and cycling in the ecosystem occupying the site.

The idea that ecto- and endomycorrhizae are beneficial in promoting absorption of nutrients from soil is firmly entrenched in the literature (Rayner, 1927; Schmidt, 1947; Kelley, 1950; Melin, 1953; Wilde, 1954; Slankis, 1958; Hacskaylo, 1959; Harley, 1959; Meyer, 1966; Gerdemann, 1968). Field observations on occurrence of ectomycorrhizae generally lead to the conclusion that this form is most frequently seen in infertile soils which implies that these structures have an important function in nutrient mobilization. In fact, Harley (1963) has stated that the ectomycorrhizal habit is an adaptation in certain species to nutrient-deficient soil.

In ectomycorrhizae forms infection is followed by morphological changes that are thought to bring about an increase in the area

of physical contact between the plant and the soil (Hatch, 1937). Although this hypothesis appears sound, it has probably never been examined experimentally. It would be interesting to compare the total absorbing surface of a nonmycorrhizal seedling with root hairs with a mycorrhizal seedling on which root hair formation has been suppressed by infection. It is well known that conifer seedlings without mycorrhizae are quite capable of absorbing nutrient elements as long as these ions are in solution. Mycorrhizal mycelia are also capable of absorbing nutrients from solution and transferring these to the higher plant (Melin and Nilsson, 1950, 1952, 1953, 1955; Melin *et al.*, 1958), but it is likely that, if absorption were the only function involved, the fungal sheath or closely grouped strands of mycelia would suffer from the same disadvantages in absorbing from solution as do closely grouped root hairs (Nye, 1966).

It seems likely, therefore, that beyond the increase in physical surface visualized by Hatch, mycorrhizae also promote solubility of soil minerals. Increased solubility in the presence of mycorrhizae may result from higher metabolic activity of mycorrhizal over nonmycorrhizal roots. This correlation has been made in studies of phosphorus uptake by conifer seedlings (McComb and Griffith, 1946; Kramer and Wilbur, 1949). Routien and Dawson (1943) investigated uptake of exchangeable nutrients by mycorrhizal and nonmycorrhizal seedlings of *Pinus echinata*. Calcium and magnesium and particularly iron and potassium were absorbed in much larger quantities from clay with low levels of base saturation by mycorrhizal than by nonmycorrhizal plants. Because development of mycorrhizae increased the average rate of aerobic carbon dioxide production of each short root from 2 to 4 times the amount recorded for uninfected roots, the authors suggested that increased salt uptake was related to an increased supply of hydrogen derived at least in part from carbonic acid. Hydrogen-saturated colloids are regarded as an efficient means for weathering minerals in soil (Keller and Frederickson, 1952; Graham, 1954).

In addition to increased carbon dioxide production, account must be taken of production of the great variety of organic substances that exude from roots or rhizospheric organisms or that result from decomposition of soil organic matter. Presumably the quantity and variety of such compounds are greater in mycorrhizal systems than in sterile systems, but differences due to mycorrhizae under field conditions would be difficult if not impossible to demonstrate. Spyridakis, Chesters and Wilde (1967) found that kaolinite and vermiculite were produced from biotite in the presence of tree seedlings growing in sand cultures. Weathering was attributed to chelation by organic matter from root sloughings.

Slankis *et al.* (1964) who studied root exudates from *Pinus strobus* found malonic, malic, oxalic, glycolic, shikimic and *cis*-aconitic acids. Also present were several sugars and amino acids and a large number of unidentified compounds. Oxalic, malonic, malic and citric acids have been identified as metabolites in glucose cultures inoculated with *Amanita rubescens* and *Rhizopogon roseolus* as well as several nonmycorrhizal inocula (Boyle, 1967;

Boyle *et al.*, 1967; Sawhney and Voigt, 1969). Oxalic acid was particularly effective as a weathering agent for biotite, muscovite, and vermiculite. In 48 hours, a molar solution of oxalic acid dissolved about 25 per cent of the potassium in ground biotite. Acid concentrations in the rhizosphere are, of course, completely unknown, but in localized instances, they may be relatively high.

A laboratory study of the growth of *A. rubescens, Boletus luteus, B. felleus* and *R. roseolus* on Hagem's agar containing particles of biotite or muscovite as the potassium source, showed that these species were all capable of absorbing potassium from the mica particles (Voigt, 1971). *R. roseolus* and *B. felleus* were more effective than the other two species. Henderson and Duff (1963) showed that common soil fungi were capable of dissolving a large variety of silicate minerals and that this ability was generally associated with acid production by the fungi. Presumably mycorrhizal fungi are also capable of producing silicate dissolving acids.

Even though many aspects of the weathering mechanism remain to be explained, there is evidence that mycorrhizal plants are able to mobilize nutrient ions from soil minerals that are relatively insoluble in extracting agents typically used in soil analysis. Rosendahl (1943) grew seedlings of *Pinus resinosa* and *Ulmus americana* in sand cultures in which orthoclase and apatite particles were used as sources of potassium and phosphorus, respectively. *Pinus* seedlings grown in sand-orthoclase media inoculated with *Boletus felleus* showed much greater growth and potassium absorption than control seedlings or those inoculated with *B. granulatus* or *Amanita muscaria.* Inoculations failed to stimulate growth of *Pinus* seedlings in the sand-apatite cultures. Seedlings of *Ulmus* grown in sand-orthoclase media were not stimulated by any fungi, but the growth of these seedlings in sand-apatite media was increased by the presence of mycorrhizal and nonmycorrhizal fungi alike.

In a similar study, Stone (1950) grew seedlings of *Pinus radiata* in a clay-sand mixture to which phosphate minerals were added. Cultures were inoculated with *Boletus luteus,* and seedlings were harvested after 11 months of growth. Seedlings grown on synthetic soil failed to demonstrate any effect of mycorrhizae on phosphorus uptake by the seedlings. The author was uncertain as to whether the mycorrhizae had been present long enough for any effect on phosphorus uptake to have been reflected in growth.

Boyle (1967) studied uptake of potassium by *P. radiata* growing in mixtures of quartz sand and biotite or muscovite mica. Seedlings were inoculated with soil suspensions or with *Amanita rubescens.* Although inoculation increased potassium uptake in some cases, the response was erratic. Again mycorrhizal development was not obvious after 8 months of growth when the seedlings were harvested. Unpublished data from a similar study at Yale in which the seedlings were allowed to grow for 16 months showed that *P. radiata* seedlings inoculated with *A. rubescens, R. roseolus* and *B. felleus* absorbed significantly more potassium from biotite and muscovite than uninoculated seedlings. Mycorrhizae were evident on inoculated seedlings.

There are several examples of field studies in which growth of trees on infertile soils was attributed to weathering ability by

mycorrhizal fungi. Wilde and Iyer (1962) described a 32-year-old plantation of *P. resinosa* on coarse glacial outwash containing unweathered feldspathic and ferromagnesian minerals. The enrichment of the surface layer in organic matter, total nitrogen, and inorganic nutrients was attributed to mycotrophic organs. Similar observations have been made with *P. rigida* (Voigt *et al.*, 1964; Voigt, 1966). Here, coarse soil particles were bound together by mycelia from mycorrhizal roots.

In a study of plant colonization of mining wastes in Pennsylvania, Schramm (1966) described yellowish mycorrhizae on *P. rigida, P. virginiana, Betula populifolia,* and *Quercus borealis* with attached golden yellow rhizomorphs. These rhizomorphs were observed to enter minute interlamellar cracks of shale where they presumably increased nutrient availability.

Nearly all examples of biological weathering associated with mycorrhizae have involved ectrotrophic forms. The vesicular-arbuscular type is, however, far more prevalent, and, in a review of recent literature dealing with this form, Gerdemann (1968) points out that their effect on plant growth appears to be similar to that of ectomycorrhizal types. He cited work which indicated that plants exhibiting vesicular-arbuscular mycorrhizae were able to absorb greater quantities of nutrients, particularly phosphorus, than nonmycorrhizal plants. This was especially true when phosphorus was supplied as rock phosphate or tricalcium phosphate, both of which are relatively insoluble. It is likely that increased solubility involved by-products of fungal metabolism.

Although the lithosphere contains the great bulk of the earth's supply of nitrogen, this source is largely inaccessible to the biosphere which obtains nitrogen largely from the atmosphere. There are several ways for nitrogen to enter the plant-soil component of the ecosystem. Rain and snow usually contain small amounts, but fixation by microorganisms is thought to be responsible for most of the nitrogen in forest ecosystems. Fixation may occur in obvious symbiosis with higher plants or may result from less obvious associations involving organisms such as *Azotobacter, Beijerinckia* or *Clostridium*. The sum of all these processes seems inadequate to explain the rates of nitrogen accumulation in many coniferous forest ecosystems (Richards and Voigt, 1965). Richards (1962, 1964) has reviewed the evidence indicating accretion of nitrogen in conifer forest-soil systems. He concluded that an annual gain of as much as 50 kg/ha is frequently attained. This is from 2 to 3 times the amount of nitrogen assumed to be added to the plant-soil system each year by recognized processes. The activities of known nitrogen-fixing agents would need to be stimulated considerably to account for the balance.

An alternative accretion could be at least partially explained if it were assumed that mycorrhizae are able to fix atmospheric nitrogen. This idea has been suggested repeatedly (Meyer, 1966; Bond, 1967; Stewart, 1967), and Melin (1925) cited Müller's opinion that *P. montana,* by means of mycorrhizae, could fix atmospheric nitrogen. Möller (1906) concluded that this was not the case, and Melin's experiments on a number of species including *P. montana* led him to the conclusion that no assimilation of atmospheric nitrogen took place either by the seedlings themselves,

with or without mycorrhizae, or by the fungi in pure cultures. He did, however, observe a slight increase in seedling nitrogen which was attributed to atmospheric ammonia absorbed by the nutrient solution.

For determining nitrogen, Melin used the Dumas method which lacks the sensitivity of the N^{15} technique. Tabak and Cooke (1968) pointed out that several studies of mycorrhizal fungi with isotopic N have suggested the possibility that some species are capable of assimilating elemental nitrogen. Bond and Scott (1955) exposed root systems of intact *P. sylvestris* to an atmosphere containing excess N^{15} for 6 days. The root systems were well-infected with mycorrhizal fungi from natural soil. No enrichment of N^{15} was detected in either intact plants or root systems, but nodulated alder plants under identical conditions showed strong enrichment. Stevenson (1959) found an enrichment of labelled nitrogen in infected roots of *P. radiata* after exposure of the whole plant to an atmosphere containing N^{15}.

Richards and Voigt (1964, 1965) exposed mycorrhizal cuttings of *Pinus radiata* and seedlings of *Pinus elliottii* to an N^{15}-enriched atmosphere for 17 days. There was a fourfold increase in total nitrogen in the substrate of *P. radiata*. No enrichment was observed in *P. elliottii* although the absolute amount of N^{15} was equal in both species, but because of the large size of *P. elliottii* plants, dilution of the isotope occurred. In another trial, *P. radiata* inoculated with *Rhizopogon roseolus* and *Amanita rubescens* fixed about twice as much N^{15} as uninoculated control seedlings. The fact that enrichment occurred in control cultures indicated that fixation was associated with soil organisms other than mycorrhizal fungi. The activity of these organisms was enhanced by the mycorrhizal fungi. The authors concluded that the site of nitrogen fixation was more likely to be the soil rather than the roots, but the organisms responsible for fixation are unknown. As Bond (1967) stated, it is obvious that more work is necessary with *Pinus* mycorrhizae, especially by means of the isotopic method in its most sensitive form. Aseptic conditions are necessary for the confirmation of any fixation apparently associated with mycorrhizae, and he suggests that isotopic examination of pure cultures of fungi concerned in *Pinus* mycorrhizae would be worthwhile.

The studies with *Pinus* presumably involved ectomycorrhizal forms. Tabak and Cooke (1968), who reviewed the literature on nitrogen fixation by fungi, cited several studies in which fixation was attributed to various species of *Phoma* which may form endotrophic associations with plants from the family Ericaceae. These results have not yet been confirmed by the isotopic technique.

In spite of the lack of indisputable evidence for fixation of nitrogen by mycorrhizal fungi in laboratory studies, there are well-known examples of the influence of *Pinus* on another tree species. Generally, the response of the affected species strongly resembles the response obtained with added nitrogen fertilizer. Stone and Will (1965) cited the case of the effect of *P. sylvestris* on *Fraxinus americana* seedlings and the response of *Cupressus macrocarpa* and *Chamaecyparis lawsoniana* seedlings in the close proximity of *P. radiata*. Richards (1962) described a similar re-

lationship between *Araucaria cunninghamii* and *P. taeda* or *P. elliottii*. Field studies with these species and with *Agathis robusta* and *P. caribaea* (Richards and Bevege, 1967) involving underplanting with legumes or application of nitrogen fertilizer showed that nitrogen is the primary limiting nutrient for the growth of the indigenous conifers. The process by which *Pinus* improved the nitrogen supply is unknown.

Stone and Will (1965) attributed increased nitrogen availability to the capacity of the pine root complex, with its associated microflora, to break down some fraction of the soil organic matter that had been inaccessible or resistant to the previous flora. The nitrogen released might be larger or smaller in amount and be either wholly immobilized in the new ecosystem or recycled in part. This hypothesis would not explain actual accretion of nitrogen unless it was assumed that refractory compounds not detected by Kjeldahl analysis were involved.

In a study of nitrogen assimilation by seedlings of *Pseudotsuga menziesii* and *Pinus radiata*, Miller (1967) found that mycorrhizal root systems had greater ability to absorb organic nitrogen compounds than did nonmycorrhizal roots. Interactions between fungus species and nitrogen source were reflected in dry matter production. Seedlings inoculated with *Amanita muscaria* grew larger than seedlings inoculated with *R. roseolus* when supplied with ammonium nitrate. When the nitrogen source was phenylalanine, histidine, glycine, or alanine, *Rhizopogon*-inoculated seedlings were larger. Seedlings inoculated with a soil suspension were significantly larger than fungus-inoculated seedlings for all nitrogen sources. Similar patterns were obtained when seedlings inoculated with different organisms were grown on substrates in which the nitrogen was supplied by leaf tissue from *Cornus florida, Alnus rugosa, Tsuga canadensis, Liriodendron tulipifera* or *Pinus resinosa*. Again, seedlings inoculated with a soil suspension were larger than fungus-inoculated seedlings. Seedlings inoculated with *Laccaria laccata* were larger than seedlings inoculated with *Cenococcum graniforme* or *A. muscaria*. As has been observed in an earlier study (Voigt, 1965b), recovery of nitrogen by seedlings was higher from coniferous leaf tissue than from hardwoods. This was probably correlated with nitrate production which was significantly higher in cultures containing conifer leaf tissue. Nitrate production in cultures inoculated with *C. graniforme* was about twice that in cultures inoculated with other organisms. These results are contrary to the findings of Carrodus (1966, 1967) who concluded that nitrate was a poor source of nitrogen for excised beech mycorrhizae.

Summary and Conclusions

In discussions of the possible beneficial effects of mycorrhizae on nutrient absorption of plants, little attention has been paid to the substrate characteristics. Differences between mycorrhizal and nonmycorrhizal plants were especially apparent on infertile soils, and on this point, there appears to be general agreement—mycorrhizae increased the capability of the infected plant to absorb nutrients. Here then may be the clue to the role played by the mycorrhizal association. Plants utilize three major forms of nutri-

ents in the growth process. Ions exist in the soil solution, adsorbed on colloidal surfaces or, ultimately, as lattice components of soil minerals. Fertile soils are characterized by high levels of ions in solution and on exchange sites. If the benefit of mycorrhizae arises from an increase in absorbing surface, it is apparent that this advantage decreases as the ion concentration increases and no particular advantage would come about in replacing root hairs, which are normally observed in fertile soils or nutrient solutions, by mycelial extensions from mycorrhizal roots or fungal mantles.

In infertile soils, concentrations of ions in solution or adsorbed on exchange sites are extremely low, and the bulk of the nutrient capital is present as components of soil minerals. Here mycelial extensions from mycorrhizal roots would be of great benefit in increasing the absorbing surface of the root. Of even greater significance is the ability of mycelia to dissolve silicates. Efficient release of ions from soil minerals would be facilitated by localization of metabolic by-products so that maximum solution can occur. Infertile soils are generally characterized by lack of organic or inorganic colloids, and as a result, the soil has no mechanism to prevent loss by leaching once solution occurs. Thus it is advantageous if the fungus producing the acids that dissolve silicates is able to absorb released ions and transmit them directly to the main conducting system of the higher plant. Mycorrhizal symbiosis fulfills both of these important functions.

This is not to imply that mycorrhizal fungi are the only organisms capable of dissolving silicate minerals. From the literature, it is apparent that many fungi and bacteria can too. It may even be that the numbers of some of these organisms as well as their total metabolic effect are enhanced by proximity of mycorrhizae (Katznelson *et al.*, 1962b). Harley (1948) has pointed out that mycorrhizae can be grouped naturally with general rhizosphere and root-region phenomena, and Wilde and Lafond (1967) have proposed new terminology to describe this concept. Considered in this light, nitrogen accretion in the continuum of which mycorrhizae are a part seems reasonable. There is no good evidence that mycorrhizal fungi are themselves directly involved in nitrogen fixation, but there is some indication that the mycorrhizal system does somehow stimulate fixation. Here again, this effect is likely to be most significant in infertile soils because of the depression of fixation by available nitrogen that is so familiar in fixation systems.

In conclusion, Harley's (1963) observation seems most to the point. He states that "one is forced to accept the fact that in a great many species of plants they (mycorrhizal roots) are the main organs of contact with the soil. In short, mycorrhizas have been selected in evolution against uninfected root-systems in a large number of species. Viewed in this way the original question of whether mycorrhizal infection is beneficial becomes secondary to the questions of how these composite organs work and how they differ in any of their functions from uninfected roots." Initiation of nutrient cycles in infertile soils is undoubtedly one of these very important differences.

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Separate No. FS-291

11.

Techniques and Procedures for Culturing Ectomycorrhizal Fungi

J. G. Palmer

Most ectomycorrhizal fungi occur in the subclass Homobasidiomycetidae, Order Agaricales (see the key of G. W. Martin, in Ainsworth (1961)). Procedures for isolation and aspects of growth in axenic culture are similar to those for the more extensively studied relatives, most of which belong in the Order Polyporales (Aphyllophorales) and are saprophytes. Symbionts commonly possess more exacting nutritional requirements, growing slowly, briefly, or not at all in pure culture. Thus Espenshade (1962) expected successful isolation from only 20% of sporophores. Oddoux (1953) reported growth of only 247 of 508 species in agar culture, and many species that grew were saprophytes. Consistent failure to grow a species in axenic culture eliminates determination of its ecological function but stimulates study of the exact requirements for growth and survival in isolation. Periodic reviews trace the refinement of old techniques and procedures (Espenshade 1962, Harley 1959, Melin 1936, 1953, 1959, and 1963).

Proof of the mycorrhizal association requires synthesis of suspects, usually in aspectic culture, i.e. inoculation of vegetative stages of host and fungus (sporophyte and mycelium in most cases) into a dixenic culture. Perfect stages of almost no ectomycorrhizal fungi develop in pure culture, and no satisfactory keys to mycelial characters exist for nonlignicolous species although a key to some boletes is available (Pantidou and Grove 1966). However, correct identifications are essential if determinations of the one or more ecological activities within a taxon are to be reliable. For instance, results of such studies in the European species of *Tricholoma*, which have been summarized by Norkrans (1950), confirm that some are ectomycorrhizal symbionts while others are saprophytes.

Isolation of Fungus Mycelium

Named isolates for use in pure culture studies and syntheses are obtained from sporophores, which can be definitely identified. Two methods are commonly used to isolate Homobasidiomycetes in pure culture, but a few to all species in some genera, especially in the Order Agaricales, grow poorly to not at all, e.g., *Amanitopsis, Boletus, Gomphidius, Lactarius,* and *Russula.*

A basidiospore-drop is the most common method used (figure 1). A portion of the hymenial surface is usually attached on the inside glass surface over an agar slant in a test tube until there is a significant drop of basidiospores, but may be also attached eccentrically to the sterile inside surface of a Petri dish lid. When test tubes are used, more than one isolation should be set up for each basidiocarp. Sterile, hard agar may be used to cement the hyme-

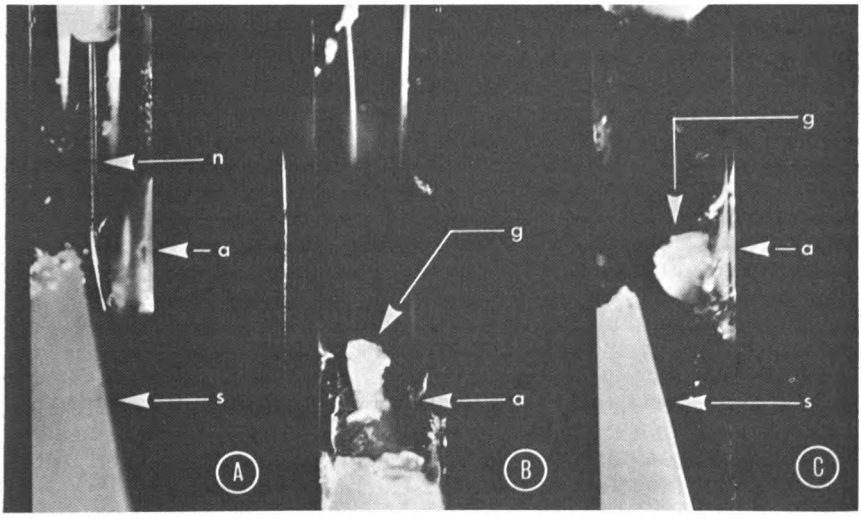

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Figure.—Preparation of sterile agar slant in test tube to catch basidiospore drop requires: A. Cutting slant (s) with sterile tip of knife or needle (n) and pushing severed agar (a) around glass to opposite side, B. placing a small piece of hymenium (g) firmly on severed agar (a), and C. sloping tube to drop spores vertically onto upper part of untouched agar slant (s). The agar (a) used to attach the hymenial tissue (g) to the glass must not touch slant (s) but permit removal of spore-bearing agar.

nium to the glass surface so that basidiospores will drop onto an undisturbed and sterile surface.

For isolation of single spores, a 2.5 percent water or nutrient agar is good because spores of most species can be washed from the surface of the agar with sterile distilled water for dilution and plating. Under high magnification, basidiospores can also be lifted off one at a time from agar in Petri dishes, but the thick tubular glass of test tubes interferes with focus and definition. More commonly, a small bit of agar with a large number of basidiospores from the spore-drop in tube or plate is transferred onto sterile nutrient agar. If possible, several such transfers are made from the area of a spore-drop. The latter increases chances for germination of one to many basidiospores free of contamination. Consequently, most isolates are polysporus in origin. In any case, hyphae with chromosomal variations may be expected. Removal of agar-bearing spores from Petri dishes is easier, but contamination by air currents, especially in the field, is more common. An equal number of test tubes occupies less space permitting larger numbers of isolations, but their use requires more expertise and a larger number of slants. Contamination of the spore-drop area can occur during insertion of the hymenium or removal of the spores, and bacteria, actinomycetes, or conidia commonly contaminate if the hymenial sporophore habitat is windy or wet. Frequently, a hymenium will produce a series of spore-drops if the hymenium is active and basidiopores mature over a period of time, and frequently only the first will be contaminated. In Petri dishes, successive drops onto new agar can be separated

by regular rotation of the lid. Basidiospores of most species will germinate between 65°F and 75°F, but a preconditioning colder temperature will induce germination in some additional species.

Isolation from Fungus Fragment

Isolation from tissue fragments of stipe or cortex in a fleshy basidiocarp is successful in a higher percent of species. Best results may be expected when fleshy fruiting bodies of the same species can be obtained in large numbers and in various stages of development. For aseptic isolation, the tissue fragments must not have been colonized by bacteria or touched by burrowing or feeding insects or animals. To reduce risk of contamination, very small fragments removed from buttons with a cool, sterile needle or knife give best results. Young sporophores collected early in the morning are good; a lower percentage of successful isolates can be made from mature specimens. In any case, the latter are needed for identification. A dry agar surface will reduce capillarity and saturation of inoculum with water causing anaerobiosis.

Initial mycelial growth from basidiospores or sporophore fragments may become visible within 72 hours but can require 2 or more weeks. Cultures stored in bell jars or open or sealed polyethylene bags will retain moisture, especially in heat, low relative humidity, or air movement. When a single locus or a circumferential ring of hyphae extends over the agar for a distance of 0.5 mm or more, many small bits (up to 1.0 mm in diameter) of hyphae-bearing agar should be transferred into test tubes on a cool, sterile needle. Large pieces increase the chances for contamination.

Contaminants are most often bacteria or actinomycetes, but some deuteromycetes, such as species of *Penicillium* and *Trichoderma,* develop conidia that have a differentiating and often characteristic color within 24–72 hours. Such a contaminant may develop on the original inoculum some time after mycelial growth of the basidiomycete is evident. Subsequent spread can be very rapid indicating that conidial germination of the contaminant, its mycelial growth, or both are inhibited during one or more stages in the development of the species being isolated.

Antibiotics, singly or in various combinations and at various concentrations, can be used to reduce growth of bacteria. Streptomycin sulfate at 80 ppm in the nutrient solution has enabled isolation of mycelium from ectomycorrhizae on pine (Zak and Bryan 1963). Several factors have favored use of penicillin and streptomycin: (1) inhibition of or lethality to ectomycorrhizal species by many antibiotics, (2) the number of experimental combinations to be tested, (3) the number of dilutions required to attain the proper concentration(s), (4) the involvement or degradation of an antibiotic in one or more preparative procedures, and (5) non-lethal or static fungal side effects such as the inhibition of fungus mycelial growth and interference with cell divisions (Cochrane 1958, p. 441).

Growth in Pure Culture

If spores are to germinate and mycelium to grow upon one or more nutrient agars, a natural or semi-synthetic medium with a pH near 5.0 (see below) is most often used. Most subcultures

develop best upon Hagem nutrient agar modified by Modess (1941, p. 16). After autoclaving, the pH is 4.6.

KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NH_4Cl	0.5 g
$FeCl_3$ (1% solution)	10 drops
Glucose	5.0 g
Malt extract	5.0 g
H_2O (distilled)	1000 cc

Subsequent modifications of this semi-synthetic formula have been numerous. Both the natural malt extract, agar first described by Melin (1936), and the commercially available dehydrated malt formulations are preferred for many Homobasidiomycetes, especially lignicolous taxa. Other natural and semi-synthetic media will result in better growth by some isolates, especially if the pH approaches 5.0 after sterilization. Thus a potato dextrose formulation may increase rate of growth of some isolates and rejuvenate vegetative growth of some staling isolates. Addition of malt extract or a casein hydrolysate will often enable initial growth or increase the rate. Among Homobasidiomycetes, growth of most symbionts will be slower than that of saprophytes, and nearly all of these fungi have a very slow rate of growth when compared generally with phytopathogenic or saprophytic moniliaceous deuteromycetes or bacteria. Another generalization may be applied to most ectomycorrhizal fungi in pure culture. If cultured simultaneously on both gel and liquid of the same nutrient formula, mycelium will grow more rapidly on the gel for reasons that are not completely understood. However, two possibilities may partially or completely explain this phenomenon: (1) Submersion in or capillary rise of liquid about hyphae will reduce oxygen tension and increase acidity in the immediate vicinity. (2) Preparation procedures may release growth stimulating or remove growth inhibiting substances in natural extracts, especially agar.

Procedures for inoculation of liquid media are described in the following paragraphs. Use of agar plugs that are 5 mm in diameter and 2 mm thick has proven most satisfactory at our laboratory. Hard (up to 3 percent) agar plugs will float on solutions in which the surface tension has not been substantially reduced, especially if the fungus will "fuzz out" (see below). However, if the agar on which the inoculum is grown contains nutrients, some will be transferred with the plug. Thus a simple carbon source such as glucose may act as a starter (Melin 1925). Such starters can enable an isolate to use one or more organics that it could not use as a carbon source in the pure state.

For some fungi, growth of each piece of inoculum to the correct diameter on nutrient solution followed by washing and inoculating in the test solution is satisfactory. If the wash solution has an osmotic pressure similar to that of the test solution, survival of inocula will be better, but compounds in classes being tested must be omitted from the sterile wash solution. This procedure requires extensive replication in order to obtain a sufficient number of pieces of inoculum.

Extension of mycelium from a nutrient agar over a barrier

(usually glass in a small, shallow beaker slightly larger in diameter than the inoculum) to water agar is also useful. Inocula can then be excised from the periphery of the colony over the water agar. Some isolates will not cross the glass edge unless covered with agar.

Since these procedures are time-consuming, most experimenters prefer nutrient agar with none or a reduced level of the carbon, nitrogen, or other compound under study.

Finally, asexual reproductive structures can be washed from the mycelia and used as inoculum. This procedure has been infrequently used because many symbionts produce no conidia, oidia, or chlamydospores, or they are difficult to separate from hyphal fragments.

Most agar plugs will float more easily with less hyphal immersion if they are aseptically excised from the starter plates, transferred to a hard agar, and allowed to remain until hyphae project around the upper periphery of the plug. At some distance between 0.5 and 2 mm, the extended hyphae will reflex down the side of the agar plug. Once hyphae reflex, the inoculum is very likely to sink or be immersed when placed in the solution. Experience with each isolate is necessary to determine if immersion reduces or stops growth, if hyphae will radiate from plugs, when they will reflex, and how much time will be required.

If a single isolate is to be used, the method of inoculation will depend upon the performance of that isolate. A study comparing several isolates requires selection of a single, compatible technique.

If the isolate grows at all in liquid culture, ultimate dry weight for most species on the same medium over equivalent time tends to be greater in standing than in shake cultures. Up to a point that must be determined for each isolate, growth (dry weight) often decreases as the number of reciprocal or rotary shakes increases.

Growth of isolates tends to be slower and less abundant upon artificial (defined) than upon natural or semi-synthetic (undefined) formulations. Thus few of several such formulae (Keller 1952, Lindeberg 1944, Mikola 1948, and others) have been widely used in physiological investigations. In carbon source utilization, we needed a synthetic medium with as few ionizing inorganic and carbon- and nitrogen-containing-compounds as possible. The pH after autoclaving was to be comparable to the Modess (1941) modification of the Hagem Medium (see tabulation p. 135). Malt extract, which possesses almost 30 percent carbon and 1 percent nitrogen by weight, and 1 percent ferric chloride solution from which $FeCl_3$ diffuses into glass, were eliminated. Over several years, a medium (table 1) which met these requirements, was formulated and tested. No precipitate forms when the compounds are concentrated in 90 percent of the water and then autoclaved. To date, isolates from 100 species of lignicolous, cellulolytic, and mycorrhizal Homobasidiomycetes have been grown on the broth formulation. The latter generally reduced the pH of this medium while a few ascomycetous fungi, mainly *Ceratocystis* spp., elevated pH when no buffering or broth replacement procedure was provided. Other workers have used this nutrient solution successfully, and most have undoubtedly elevated the carbon equivalent per liter.

Assay of comparative vegetative growth as a function of one particular factor is complicated if that factor inhibits or stimulates

Table 1.—*Synthetic nutrient formulation of pH 4.6 for pure culture of Homobasidiomycete mycelium*

Additive	Amount per liter	Equivalent per liter	
		Carbon	Nitrogen
D-Glucose	5 g	2.0 g	0
NH_4Cl	500 mg	0	130.9 mg
KH_2PO_4	500 mg	0	0
$MgSO_4.7H_2O$	500 mg	0	0
Thiamin (Vit. B)	1 mg	427.4 μg	166.1 μg
Biotin (Vit. H)	5 μg	2.5 μg	0.6 μg
Micro Elements [1]	2 ml	0	151.0 μg

[1] Micro Element Solution (Lilly and Barnett 1953, p. 9: $Fe(NO_3)_3.9H_2O$ (723.5 mg), $ZnSO_4.7H_2O$ (439.8 mg), and $MnSO_4.4H_2O$ (203.0 mg). Dissolve in 700 ml distilled H_2O. Acidify with H_2SO_4 until solution clears. Dilute to 1000 ml. (each ml contains 100 μg each of iron and zinc and 50 μg of manganese.)

germination of conidia, oidia, or chlamydospores. An example has been recorded for *Cenococcum graniforme* by Mikola (1948), who did not experimentally differentiate the effect upon vegetative growth of mycelium from effect upon germination of chlamydospores. This problem is common in spore-forming isolates, but fewer mycorrhizal than lignicolous species produce asexual reproductive structures in axenic culture. Perhaps compounds such as fungicides that suppress germination but not mycelial growth could be used in some experiments.

Since some workers may be unacquainted with these asexual structures, each is discussed below. Those of Basidiomycetes have been most clearly defined by Snell and Dick (1957). Chlamydospores and oidia may be either intercalary or terminal; conidia are usually terminal on conidiophores as drawn by Davidson et al. (1942) and Maxwell (1954). Drawings of chlamydospores (Davidson et al. 1942, Davidson and Campbell 1943, Lombard and Gilbertson 1965) and oidia (Brodie 1936, Lombard and Gilbertson 1965, McKay 1959) indicate similarities that make differentiation of immature stages difficult. Photomicrographs are few, but oidia have been photographed by Hanna (1938) and chlamydospores by Harmsen (1967) and Nobles and Nordin (1955). Oidia are generally the smallest of the spore types having a minimal width or diameter of 1-2 microns. They tend to be rectangular in longitudinal view and oval in cross-section. Conidia are intermediate in size and variable in shape but are usually oval to ellipsoidal. Both are thin-walled. The ellipsoidal or oval thick-walled chlamydospores are largest and can measure up to 25-30 microns in diameter or length. Since size of such structures in a species can be similar (see *Pholiota adiposa* in Davidson et al. 1942, p. 26), knowledge of the ontogeny and morphology of mature structures may be essential.

Growth Factors Affecting Ectomycorrhizal Fungi

Light: Almost no studies have reported the effects of various wave lengths upon ectomycorrhizal fungi in pure culture or in synthesis with the host. Indirect natural light influenced color formation and strong light inhibited growth of mycelia of lignicolous saprophytes (Davidson et al. 1942), but those of mycorrhizal and lignicolous species of *Tricholoma* did not respond (Rawald 1962).

The probability that light will reach and affect isolated mycelia or mycorrhizae in soil is slight in any case. The general absence of obvious growth responses and sporophore initiation by mycelia of ectomycorrhizal fungi in pure culture probably accounts for the lack of published information.

Temperature: A constant temperature between 20°C and 25°C is satisfactory for general culture. Optimal temperatures for mycelial growth lie between 18°C and 27°C for the majority of species (Harley 1959, p. 62). For many, growth ceases above 35°C and below 5°C (Hacskaylo et al. 1965). In most cases, the optimum for an isolate is close to its maximum. Of interest is the fact that most isolates studied to date originated in northern latitudes, especially Sweden, Germany, Austria, Japan and the United States. No studies record the influence of daily temperature variations upon mycelial growth, but mycorrhizae formed by these soil-inhabiting fungi experience more of a seasonal (Zak and Bryan 1963) than a diurnal fluctuation.

Gases: Limited available data indicate that ectomycorrhizal fungi are obligate aerobes. Melin (1923) attributed reduced growth of mycelium submerged in broth to lowered oxygen tension, and other investigators have agreed. Though cotton plugs are generally thought to reduce oxygen and increase carbon dioxide tensions, growth of some isolates was similar with or without air movement through tubes (Rawald 1962). In a recent review, Melin (1953) concluded that these fungi cannot fix atmospheric nitrogen. No one has varied tensions of gases other than oxygen and carbon dioxide nor studied multiple mixtures.

Growth regulating compounds: Some compounds likely to be involved in culture of ectomycorrhizal fungi will regulate (increase or decrease) metabolism or contain moieties for which the fungus is heterotrophic. Requirements for such compounds by fungi have been presented in tabular form (Norkrans 1950) and indicate that growth of basidiomycetes will most likely be stimulated by or dependent upon two vitamins. Of these, aneurin (Vit. B_1 or thiamin) is required by more species than biotin (Vitamin B). In other words, available data indicate that thiamin must and biotin ought to be added to a satisfactory synthetic medium. Both may be sterilized by autoclaving in the nutrient solution. Biotin is apparently stable; thiamin may disintegrate into its pyrimidine and thiazole components, one or both of which will be active (Melin and Nyman 1941, Norkrans 1950).

A few isolates have been reported to be deficient for inositol, nicotinic acid, and pantothenic acid (see Harley 1959). Complexes of vitamins may either stimulate or depress growth (Melin 1953, Mikola 1948) but have not noticeably increased the number of fungi introduced into pure culture for the first time (Melin 1954, Mikola 1948, Norkrans 1950).

Amino acids at or below one gram per liter of nutrient solution may stimulate or depress growth. Sometimes inhibition occurs at concentrations only slightly higher than that required for optimum growth (Melin 1963, Mikola 1948, Norkrans 1953). Fractions of casein hydrolysate, which contains amino acids at determinable levels (Norkrans 1950), were metabolized by isolates of *Cenococ-*

cum graniforme, and some in low concentration stimulated germination or growth of chlamydospores (Mikola 1948).

Seeds and roots attached to the living plants of many herbaceous and arboreal phanerograms, especially conifers, produce an exudate which markedly increases growth of culturable ectomycorrhizal fungi and enables two-organism (dixenic) culture of some fungus species that cannot be cultured alone (Melin 1923, Melin 1925). No individual or combination of vitamins or amino acids has equalled this stimulation, but mixtures of amino acids and B-vitamins have increased total mycelial growth of some isolates in the presence of living roots attached to stems (Melin 1953, Melin and Das 1954). Repetitive successful pure culture of many ectomycorrhizal fungi will be improbable until the one or more stimulatory materials or other compounds with analogous effects are discovered.

Nitrogen sources: The early postulation that ammonium salts of inorganic acids, especially those of hydrochloric and sulfuric acids, would be especially usable sources of nitrogen for a wide range of mycorrhizal fungi (Melin 1925) has been confirmed. If used as sole source, removal of ammonium cations causes a very rapid fall of the hydrogen ion concentration to the tolerable minimum (see hydrogen ion concentration below), even if slightly buffered. Urea and nucleic acids, which were suggested as satisfactory general organic sources (Melin 1925), eliminate this problem but are difficult to obtain in an inexpensive pure form and can be used by fewer species of fungi. Asparagine, peptone, and other organic compounds containing nitrogen may also provide an additional source of carbon. Metabolism may be altered by each change in an environmental factor, variation in the amount of any nutrient chemical, or addition of a fungicide or other growth regulating compound. For example, utilization of ammonium tartrate as sole source of nitrogen by *Cenococcum graniforme* was considerably less when an oligosaccharide was the carbon source than was utilization in the presence of glucose (Keller 1952). Such complications have encouraged use of ammonium salts of inorganic acids, especially NH_4Cl, as sole source of nitrogen in axenic culture of ectomycorrhizal fungi.

Altered degradation products, especially from organic compounds, and interactions among substances may create configurations that will be growth regulatory instead of utilizable in nature or vice versa. Thus new combinations that affect metabolism are regularly created in any culture system, but pure culture systems generally lack most of the adsorption, buffering, structure, etc. that influence metabolism in soil.

Inorganic ions other than nitrogen: In fungus nutrition, an essential metal may be (1) essential to every fungus (K, Mg, Fe, Zn, Mn, Mo, Cu); (2) essential for some but not for others (Ca); or (3) required by a very few fungi, often only under certain conditions (Ga, Sc, V) (Lilly 1957). Nonmetals are also required (H, C, N, O, P, S) (Ainsworth and Sussman 1965). The magnesium sulfate ($MgSO_4 \cdot 7H_2O$), dihydrogen potassium phosphate (KH_2PO_4), ferric chloride ($FeCl_3$), ammonium chloride (NH_4Cl), and glucose of the Hagem formulation (Modess 1941) supply all essential elements which are required in quantity and

probably an excess of sulfur. The manganese, molybdenum, and copper trace requirements must be supplied as contaminants in a compound such as malt extract, diffusates from one of the containers, or may be substituted by an unknown ion. A complete trace element solution, such as that of Lilly and Barnett (1953), will provide all essential trace elements for vegetative growth. Which elements are essential and under what conditions as well as phenomena of biological substitution have not been determined for ectomycorrhizal fungi. It is possible but unlikely that one or more specific but hitherto unsuspected elements are required for growth of some unculturable species or for continuing culture of some short-term survivors.

Carbon sources: The early hypothesis that glucose would be the only carbon source with which dry weight of true mycorrhizal fungi would markedly increase (Melin 1925) must be modified to include other simple carbohydrates, especially mannose (Keller 1952, Norkrans 1950). The hexahydrose alcohol, mannitol, and the polysaccharides, pectin and dextrin, are also efficiently used by a few species (Keller 1952, Melin 1953, Mikola 1948, Norkrans 1950). Mannitol is widely distributed in plants, especially in exudates (Robinson 1967). Beech mycorrhizae have been shown to possess soluble glucose, fructose, sucrose, trehalose, two inositols, and mannitol, but nonmycorrhizal roots lack both this polyol and trehalose (Lewis and Harley 1965). These data suggest that mycelium of ectomycorrhizal fungi may survive for periods of time in pockets near injuries to roots or near roots, the exudate from which contains mannitol. If this alcohol should stimulate germination of spores, especially basidiospores, distribution of and host invasion by some ectomycorrhizal species is closer to understanding. Occurrence would not be restricted to the vicinity of host roots if mannitol is the sole stimulator. Many fungi contain mannitol (see Cochrane 1958), and Lewis and Harley (1965) suggest that mannitol might be released in soil by decomposing fungi or enzymatic conversion of glucose, fructose, and mannose.

No experiments have examined the use of a large number of purified carbon sources under similar conditions nor have a large number of fungi been exposed to any but glucose. Few oligosaccharides and almost no polysaccharides are efficiently used in pure culture (Norkrans 1950, Rawald 1962) though most ectomycorrhizal isolates apparently produce cellulase (Lyrl 1963). Pectic substances are common in primary cell walls and intercellular cement (Robinson 1967). Since ectomycorrhizal fungi grow intercellularly within the cortices of host roots, utilization of pectin is probable. Use of polysaccharides is often increased by addition of a small amount of a readily metabolizable carbohydrate (Melin 1925, Mikola 1948), which has been termed a "start sugar" (Norkrans 1950).

In summary, glucose, mannose, mannitol, dextrin, asparagine, or combinations of these compounds will prove satisfactory carbon sources for most ectomycorrhizal fungi.

Hydrogen ion concentration: Modess (1941) determined the pH requirements of a large number of ectomycorrhizal Hymenomycetes. Optima varied between 3.5 and 5.9 with little growth of any species below 2.5 or above 6.9. Tolerances tended to be highest for

boleti (mostly present species in *Suillus*) and lowest for amanitas, but definitive data have been recorded for relatively few species in any other groups. In fact, the only other extensive study of this specific factor also recorded optima (nearer pH 4.0) for boletes (Hübsch 1963). Thus acidophilic media with an initial pH near 5.0 will enable pure culture of the widest range of ectomycorrhizal fungi.

Helpful Procedures and Techniques

1. Date, specific place (at least county, state, or country), altitude, ecological associates, host, and substrate should be recorded at the time of sporophore collection. Color(s) of the sporophore from a stated color manual must be recorded as soon as possible. Photographs, especially color, must be accurately made, exposing in light of the correct Kelvin rating for the film, before color fades or shrinkage occurs.

2. Correct identification of fungus and host plant is essential. The name of the authority who verified the identification, the collector's name(s), collector's number, the herbarium into which the sporophore was placed, and the one or more culture collections into which the isolate was deposited are essential if other investigators wish to use the isolate.

3. The source of each culture used in an experiment as well as the number assigned by the sender, if other than an original isolation, should be reported. Subcultures of borrowed isolates should not be sent out with a new number but should be marked with the number under which it was received and with the name of the contributor.

4. The possibility that a series of carbon or nitrogen sources, vitamins or other growth regulating compounds, "start" glucose or antibiotic compound, or a variety of some other class of soluble compounds will initiate or stimulate growth can be tested by placing one or more upon glass-fiber or cellulose discs (antibiotic type), dried, added singly or in various combinations on nutrient agar lacking the compound(s) and then inoculated with the isolate. Testing of soluble compounds is obviously more practical. Presterilization is often unnecessary with fungicides. In the case of carbon sources, the isolate will often grow toward the utilizable compound(s) and the rate of growth will be proportional to the utility.

5. Predried and weighed glass-fiber filter papers are better for mycelial dry weights than cellulose papers, which are hygroscopic. However, the former tear more easily, especially when wet. They should be washed, oven-dried, and weighed before use and may be marked centrally with a felt pen on the side that will be on the plate of the Buchner funnel before drying and weighing. Extraction of water from mycelium by vacuum is quicker and more satisfactory when glass wool is placed under the filter paper and the moist filter paper is sealed around the vertical edge of the funnel rather than on the base. The diameter of the filter paper should be greater than the diameter of the funnel by at least 2 cm.

6. Solutions of natural extracts should be sterilized through filters having a pore size of 0.18 microns or smaller to eliminate all bacterial structures. Many of these contaminants will not grow until the filtrate is added to complete the nutrient solution.

7. There is little or no guarantee against structural alteration during sterilization of soluble macro-molecular and most insoluble compounds. The most common methods have used heat, especially autoclaving, for sterilization. Heat-labile materials may be degraded, and volatile compounds lost (Greathouse and Rigler 1941, Purvis et al. 1966, p. 223–235). Ethylene and propylene oxides have been reported less damaging (Hansen and Snyder 1947, Klarman and Craig 1960). In the liquid state, these agents degrade plastic (Goss and Marr 1963) or leave toxic residue (Smith 1965). Our evidence indicates that residues occur with both and are toxic to some isolates. More satisfactory and rapid methods are needed. For example, short wave ultra-violet light (260 mμ), which does not pass through glass and travels in straight lines, might be useful for sterilization of powdered compounds that can be kept in constant motion and are undegraded by the radiation.

8. Mycorrhizal isolates can often be revitalized by selective transplantation of the youngest hyphae onto fresh Hagem, malt extract, or potato dextrose agar. Variation of temperature above and below 20°C may also help. A more satisfactory but elaborate method is pure culture synthesis with an acceptable host and reisolation from mycorrhizae. Revitalization is possible but not necessarily probable if the fungi are inoculated on agar in the vicinity of roots from sterile intact seedlings according to the method of Melin (Melin 1925, Melin 1954, Melin and Das 1954).

Summary

Most identified ectomycorrhizal fungi are in the subclass Homobasidiomycetidae, Order Agaricales. Failure to fruit in aseptic culture has required isolation of suspect fungi from identifiable sporophores. Procedures are described. Such isolates can then be synthesized with a host. The most common and successful nutrient formulation has been the semi-synthetic Hagem as prepared by Modess (1941) and modified by many investigators. Malt extract has been the most successful natural medium, and as a supplement, often increases growth on undefined nutrient media. Casein hydrolysate is effective for fewer species. Synthetic (defined) formulae must possess potassium, magnesium, iron, zinc, manganese, molybdenum, copper among metals and hydrogen, oxygen, phosphorus, sulfur, carbon, and nitrogen among non-metals. The best carbon sources are simple sugars including glucose, mannose, and mannitol. The best nitrogen sources are inorganic ammonium salts, but asparagine, peptone, and nitrate salts are used by some fungi. The medium must contain thiamin (vitamin B_1 or aneurin) since most ectomycorrhizal fungi are heterotrophic for vitamin B_1 and should contain biotin. Inositol, niocotinic acid, and pantothenic acid also increase total growth of some species. Most satisfactory media have a pH of 4.7-5.0 after sterilization. A synthetic formulation on which more than 100 homobasidiomycetous fungi have grown successfully in liquid culture is described. Most ectomycorrhizal fungi examined to date are aerobic, are insensitive to light, and grow best at a constant temperature between 20°C and 25°C.

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Separate No. FS-292

12.

Physiology of Vesicular-Arbuscular Mycorrhizae[1]

Lynn E. Gray

The endomycorrhizae are divided into two types: (1) those formed by septate fungi and (2) those formed by aseptate fungi. The latter type (also known as vesicular-arbuscular or VA mycorrhizae) are the most common and occur in a wide range of plant habitats. They grow throughout the world in forest soils (Baylis, 1959; Baylis et al., 1963; Clark, 1963), sand dunes (Nicolson, 1960), and other soils (Gerdemann, 1955, 1961; Gerdemann and Nicolson, 1963; Johnson, 1949; Mosse, 1953; Mosse and Bowen, 1968). Endomycorrhizae are of particular interest because of their beneficial effect on plant growth and because of the number of economically important forest trees and agricultural plants on which they occur. Some of the important plant species which have VA mycorrhizae are: corn, red clover, soybean, cotton, tobacco, peas, apples, citrus, tuliptree, sweet gum, maple, and cottonwood.

Characteristics of VA Mycorrhizae

External Characteristics

VA mycorrhizae have an extensive hyphal network that extends from the root as far as 1 cm (Mosse, 1959; Nicolson, 1959). The hyphae may grow on the root epidermis, but, do not form a hyphal mantle around the root. External hyphae are thick-walled and variable and irregular in shape (Gerdemann, 1955; Mosse, 1959; Nicolson, 1959). Vesicles and thick-walled spores are produced in the soil (Gerdemann, 1961, 1965; Mosse, 1956).

Internal Characteristics

Hyphae grow intracellularly or both intra- and intercellularly in the root cortex. These hyphae form coils within the cortical cells and loops between and within cells. In some cases, the fungus completely colonizes the cortical region of the root, but it does not invade the endodermis, stele, or meristem. The fungus produces haustorial-like structures called arbuscules within the cortical cells. Arbuscules are usually produced terminally on hyphae, but in some cases, they may form laterally on hyphae that grow from cell to cell. It is believed that these structures function in the exchange of nutrients between the host cell and fungus.

Fungi-Producing VA Mycorrhizae

It has been shown experimentally that *Endogone* species produce vesicular-arbuscular mycorrhizae. Mosse (1953) found small sporocarps of an unnamed *Endogone* species attached to mycorrhi-

[1] Cooperative investigations of the Crops. Res. Div., Agr. Res. Serv., U.S. Dep. Agr., and the Ill. Agr. Exp. Sta. 2# Pub. No. 575 of the U.S. Reg. Soybean Lab., Urbana, Ill.

zal strawberry roots. Using spores of this fungus as inoculum, she demonstrated that this fungus produced VA mycorrhizae. Gerdemann (1955) and Gerdemann and Nicolson (1963) extracted a number of different *Endogone* spores from soil and showed that they could produce mycorrhizae. Recently, they classified and named a number of common *Endogone* species (1968).

Endogone species which form VA mycorrhizae have a very broad host range. *Endogone fasciculata* forms VA mycorrhizae on corn and tulip trees (Gerdemann, 1965); *E. mosseae* forms VA mycorrhizae on corn, tuliptree, sweet gum, soft maple, and cottonwood (Gray, 1964). Other *Endogone* species maintained on other plant species have been shown to form VA mycorrhizae (Daft and Nicolson, 1966; Mosse, 1956).

Investigations Using VA Mycorrhizae

One of the problems in studying VA mycorrhizae has been the difficulty of obtaining pure cultures of the fungus (Gerdemann, 1955; 1961). Therefore, much of our knowledge of VA mycorrhizae has been obtained with "pot cultures" consisting of a single *Endogone* species maintained on the roots of living plants grown in pasteurized soil. Spores of the fungus produced in these pot cultures have been used as inoculum for host range and plant growth studies (Daft and Nicolson, 1966; Gerdemann, 1955, 1965; Gray, 1964). This technique does not eliminate the possibility that contaminating microorganisms are responsible for some of the observed growth differences attributed to mycorrhizal infection. Lack of pure cultures has seriously limited the techniques that can be used to study plant growth and nutrient absorption by mycorrhizal plants. It has also handicapped studies on the physiology and metabolism of the fungi. However, many workers have used various methods to inoculate plants that compensate for the possible growth effects induced by contaminating microorganisms (table 1).

Table 1.—*Inoculation methods using* Endogone *species*

Inoculum	Control	Reference
Surface sterilized spores	Sterile substrate	Mosse (1956)
Spores or sporocarps	Wash-water from sporocarps	Gerdemann (1964)
Infected roots	Wash-water from infected roots	Murdock *et al.* (1967) and Gray (1964)
Do.	Autoclaved roots	Gerdemann (1965)
Unsterilized soil	Uninfected plants from soil transplanted to experimental soil	Baylis (1959)

Effects of VA Mycorrhizae on Plant Growth

Asai (1943) compared the growth of mycorrhizal and nonmycorrhizal plants in autoclaved soil and in autoclaved soil to which nonsterile soil was added. It is quite probable that other soil microorganisms in the soil inoculum could have been responsible for some of the increased plant growth. The mycorrhizal plants grew better when inoculated, but plants which were normally nonmycorrhizal grew equally well in either soil treatment. Clark

(1963) used surface-sterilized mycorrhizal roots as inoculum for tulip trees grown in soil fumigated with methyl bromide. After 12 weeks, the fresh weight of infected plants was six times greater than that of uninfected plants. Murdoch et al. (1967) demonstrated that Sudan grass grown in pots containing mycorrhizal corn roots produced greater dry matter and contained a higher percentage of phosphorus than uninoculated plants. However, the filtrate from the infected corn roots stimulated growth of nonmycorrhizal Sudan grass. This growth was less than that of the mycorrhizal plants, perhaps because of the microorganisms on the inoculum. Meloh (1963) obtained increased growth of corn and oat seedlings grown in sand culture and inoculated with a mycorrhizal fungus. Peuss (1958) using mycorrhizal roots as inoculum, obtained increases in the growth of mycorrhizal tobacco grown in a subsoil and in a soil which had been fallow. When fertilizer was applied to the subsoil at three levels, the mycorrhizal plants grew better than the controls at all applications. Also, mycorrhizal plants in nutrient solutions adjusted to pH 4, 5, and 7, had better growth than did uninfected plants.

Baylis (1959) grew *Griselinia littoralis* seedlings in soil containing a VA mycorrhizal fungus. The seedlings were then transferred to steamed soil low in phosphorus and other nutrients. Some of the seedlings became mycorrhizal and grew better than those which did not become mycorrhizal. Growth of the nonmycorrhizal plants was poor, and eventually stopped. Mycorrhizal plants had a higher percent of phosphorus and potassium and a lower percent of nitrogen. Baylis (1967), in further studies on the effect of VA mycorrhizae on the growth of several woody plants of New Zealand, presented evidence that steaming of soil had a slightly favorable effect on plant growth. This effect was attributed to an increase in available phosphorus. However, partial sterilization of the soil had a deleterious effect on plant growth that was associated with the destruction of the mycorrhizal fungus. If soluble phosphorus was added to steamed soil, the nonmycorrhizal plants grew as well, or better than, mycorrhizal plants grown in unheated soil with the same amount of added phosphorus. He concluded that in New Zealand forest soils, which are normally low in available phosphorus, mycorrhizae are essential for the uptake of adequate phosphorus for growth.

After it was shown that spores and sporocarps of mycorrhizal *Endogone* maintained in "pot cultures" could be used as inoculum, more well-defined inoculation techniques were used to study the effects of mycorrhizae on plant growth. Mosse (1957) used sporocarps of *E. mosseae* to inoculate apple seedlings and cuttings grown in an autoclaved soil-sand mixture. In a series of five experiments, the mean size of mycorrhizal plants was greater than the controls. Mycorrhizal plants had a higher potassium, iron, and copper content and a lower manganese content than the nonmycorrhizal plants.

Gerdemann (1964) inoculated corn grown in steamed soil infested with sporocarps of an Illinois isolate of *E. mosseae*. Water which contained the sporocarps was decanted and added to the soil in the control pots. This procedure has become a standard practice for introducing contaminating microorganisms into the

control soil. The mean dry weight of tops of mycorrhizal plants was 4 times greater than that of the controls. A soil test at the end of the experiment showed that the mycorrhizal plants had removed significantly more soil phosphorus than the nonmycorrhizal plants. Tuliptree seedlings inoculated with an Illinois isolate of *E. mosseae* became mycorrhizal and showed significantly greater growth than the nonmycorrhizal plants. The mycorrhizal plants contained significantly more phosphorus than uninfected plants (Gray, 1964). Holevas (1966) grew mycorrhizal and nonmycorrhizal strawberry plants in a soil low in phosphorus and in the same soil with added KH_2PO_4. In the low-phosphorus soil, the mycorrhizal plants absorbed more phosphorus and had a higher dry matter production than the nonmycorrhizal plants. There was no difference in phosphorus uptake and growth of mycorrhizal and nonmycorrhizal plants in the soil in which phosphate was added.

There is increasing evidence that VA mycorrhizae affect plant utilization of less available forms of phosphorus such as rock phosphate, bonemeal, and tri-calcium phosphate. Daft and Nicolson (1966) used three separate *Endogone* species to inoculate plants grown in sand and watered with a nutrient solution. They obtained the largest increase in growth in mycorrhizal plants under low phosphorus availability. Murdoch, Jackobs, and Gerdemann (1967) found that corn growing in steamed soil inoculated with *E. mosseae* did not grow significantly better than control plants when both were supplied with superphosphate or mono-calcium phosphate. However, when rock phosphate or tricalcium phosphate was used, the mycorrhizal plants were larger and contained a higher percentage of phosphorus than the nonmycorrhizal plants.

The greater phosphorus absorbing capacity of mycorrhizal plants compared to nonmycorrhizal plants has been shown using P^{32}. Gray and Gerdemann (1967) transferred 95-day-old mycorrhizal and nonmycorrhizal sweet gum seedlings to individual containers of steam-sterilized soil. After an additional 40-day growth period, 14 μc of P^{32} were injected into each pot. The plants were exposed to the P^{32} for 6 days; then the radioactivity in leaf pieces of the individual plants was determined. The radioactivity in the foliage of mycorrhizal sweet gum was 15 times higher than that of the controls. Mycorrhizal sections of onion roots contained much larger quantities of P^{32} than did nonmycorrhizal segments or root tops (Gray and Gerdemann, 1969).

Application of a fungicide reduced P^{32} accumulation in mycorrhizal onion root segments, suggesting that the fungus was responsible for the increased capacity of mycorrhizae to absorb phosphate (Gray and Gerdemann, 1969). Bowen (1968), using P^{32} with both pure two-member cultures and with "pot grown" plants of onion and clover, showed that mycorrhizal plants absorbed nearly twice as much phosphate as uninfected plants. With autoradiography, they demonstrated that the mycorrhizal portions of roots showed the most radioactivity and the radioactivity was concentrated in fungal structures. This latter work indicates that the fungus is directly responsible for the increased phosphate uptake by mycorrhizae. Detached mycorrhizal nodules of *Agathis australis* absorbed larger amounts of P^{32} than nonmycorrhizal nodules (Morrison and English, 1969).

Conclusion

There is increasing evidence that VA mycorrhizae can increase plant growth by: (1) increasing nutrient content of plants through greater nutrient absorption of infected roots and (2) by increasing utilization of less available forms of phosphorus.

Additional information is needed on what effect VA mycorrhizae have on plants growing in fertile soil under the very best growing conditions. Field crops in Illinois may be highly infected even when they are growing in very productive soils. Any beneficial or harmful effect of the mycorrhizae on the individual plants could be of considerable importance.

Renewed attempts should be made to isolate and culture the mycorrhizal *Endogone* species and determine their cultural characteristics and their physiological processes. More work is needed on the ecology of VA mycorrhizal fungi and their distribution under various soil and fertility conditions. We know VA mycorrhizae are widely distributed in nature, but we have little information on the environment, soil, and plant species that affect their distribution and survival.

Much more information is needed on how VA mycorrhizae affect plant growth. Some unanswered questions are: (1) How does the fungus affect nutrient absorption? (2) Does the fungus produce some stimulatory metabolite that increases plant growth? (3) Are mycorrhizae essential under natural conditions for plant nutrient absorption? (4) What effects do mycorrhizae have in plant disease resistance?

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Separate No. FS-293

13.

Formation of Ectomycorrhizae of Forest Trees in Relation to Light, Carbohydrates, and Auxins

V. Slankis

Introduction

In 1900, Stahl postulated that the presence of mycorrhizae of forest trees is a manifestation of nutritionally poor soils. He believed that in such soils the host plant cannot compete with other soil microorganisms for the scarce essential nutrients without the aid of the symbiotic fungus. Later, Melin (1923) stressed that, in order to enter the roots of the host plant, the symbiotic fungus must have a certain virulence and that the degree of this virulence is conditioned by the nutritional properties of the soil. That poor soils are more stimulating for ectomycorrhiza formation than soils with easily available nutrients was later confirmed by Melin (1925), McComb (1943), Routien and Dawson (1943), and McComb and Griffith (1946).

Hatch (1937), Björkman (1942), Mitchell, Finn, and Rosendahl (1937), Harley (1959), Hacskaylo (1957) and many other investigators, including researchers in Russia (see Shemakhanova, 1962), provided convincing experimental evidence that nutritional properties of the soil do indeed greatly influence the formation of symbiotic relationship. Hatch (1937), experimenting with several species of pine and using different cultivation methods, concluded that four nutritional elements are important in mycorrhiza formation, namely, nitrogen, phosphorus, potassium, and calcium. He found that mycorrhizae were abundant if there was an imbalance in the availability of one or more of these elements and rare if there was an ample supply. In Hatch's opinion, susceptibility to infection by mycorrhiza-forming fungi is controlled indirectly by the internal concentration of nutrient elements in short roots. Björkman (1942) found that in addition to inorganic soil nutrients, particularly nitrogen and phosphorus, the formation of mycorrhizae is also greatly influenced by light intensity. At light intensities below 25 percent of full daylight, mycorrhizae frequency decreased considerably, and, at a light intensity as low as 1/16 of full daylight, mycorrhizae no longer formed.

Ectomycorrhiza formation in relation to carbohydrates

By carrying out chemical analyses on reducing soluble sugar content in the roots, Björkman found readily reducing substances only in the roots of seedlings grown under those nutritional and light conditions which had stimulated mycorrhiza formation. In view of these findings, he postulated that the presence of free soluble sugars in the roots is the sole factor necessary for ectomycorrhiza formation. According to his theory, the symbiotic fungus, seeking soluble carbohydrates, will enter the roots and form a

symbiotic relationship with the host plant only if a surplus of these sugars is available in the roots.

Although Björkman's carbohydrate theory on ectomycorrhiza formation was generally accepted for more than two decades, experimental data derived from more recent studies on forest trees reveal that the formation of this symbiosis does not necessarily follow the principles expressed in this theory and, in fact, that this process appears to be far more complex. Of interest in this respect are studies by Meyer (1962, 1965) on the relationship between soil fertility, mycorrhiza frequency, and soluble sugar content in the roots of *Fagus sylvatica* seedlings. Like Björkman, Meyer found that as the amount of soluble sugars in the roots increased, the number of mycorrhizae also increased. However, Meyer could not confirm that part of the carbohydrate theory in which it is stated that an increase in nitrogen and phosphorus availability results in a decrease in mycorrhiza frequency. His experiment suggested just the opposite. Potted seedlings, grown in already nutritionally rich and microbially active soil had more soluble sugars in their roots and produced more mycorrhizae after they had received additional nitrogen and phosphorus fertilization. From this, Meyer concluded that an increase of soluble sugars in the roots is not the cause, but the effect of the symbiotic association.

That soluble sugars can be present in the roots despite extreme variations in the availability of nitrogen and phosphorus becomes apparent also from results obtained in joint studies of the Biology Department of Queen's University and our laboratory (Lister *et al.*, 1968). In these studies, white pine (*Pinus strobus* L.) seedlings, grown in a forest nursery for three years, were used. They were carefully excavated in the spring, and only those with abundant mycorrhizae were selected. Before potting, their root systems were gently but thoroughly rinsed to remove adhering soil particles. The cleaned seedlings were planted in paraffin-soaked clay pots filled with washed granitic sand. The potted seedlings were randomly divided into ten nutritional groups (table 1) and transferred outside to a specially-built concrete frame covered by a transparent plastic roof which reduced the full sunlight intensity by almost 50 percent and provided protection from rain. The seedlings were grown in this frame, with their pots suspended above the ground. By means of a special insulation arrangement, their root systems were subjected to temperatures which differed very little from the ground temperatures at a depth of 22 cm.

Table 1.—*Distribution of* C^{14} *among the ethanol-soluble sugars in the roots of* Pinus strobus *L. seedlings grown on various levels of nitrogen and phosphorus. Duration of photoassimilation, 8 hr* [1]

P mg/l	0		173				692			
N mg/l	53	265	0	2.5	53	265	0	2.5	53	265
	Percent of total C^{14} in sugar fraction									
Sucrose	55.0	53.0	85.4	78.8	71.1	57.0	82.6	91.4	86.2	70.8
Glucose	14.2	10.9	4.8	7.4	12.1	17.2	4.9	2.9	5.7	8.6
Fructose	13.5	11.0	4.7	7.0	10.8	16.3	4.5	2.6	5.1	8.1
Raffinose	14.9	23.2	4.7	5.8	4.8	6.9	7.7	2.8	2.4	11.3
Unknown	2.4	1.9	0.4	1.0	1.2	2.6	0.3	0.3	0.6	1.2

[1] From Lister *et al* (1968).

The basic nutrient solution and six combinations of nitrogen and phosphorus concentrations were the same as Björkman used in his experiment and which he designated as N_0P_1, N_1P_1, N_5P_1, N_0P_4, N_1P_4 and N_5P_4 (see Björkman 1942, p. 133). In the remaining four nutritional groups, only nitrogen or phosphorus was added to the basic solution. To keep the soil sufficiently moist, the pots were irrigated with these solutions every second day. The excess of nutrients was efficiently removed through a specially arranged drainage device.

After 13 weeks of cultivation under these experimental conditions, the seedlings were brought into the laboratory and their shoots were enclosed individually in a transparent plastic chamber. Under a light intensity of 2,500 ft-c, a prescribed amount of $HC^{14}O_2$ was released in the chamber. After 8-hour photoassimilation, the root systems were separated, cut into small pieces, and their contents extracted in 80 percent ethanol. Resin columns, as described by Shiroya *et al.* (1962), were used to separate the ethanol solution into amino acid, sugar, and organic acid fractions.

During the 8-hour photoassimilation period in all nutritional groups, 95 to 99.8 percent of C^{14} translocated from shoots to roots was present in the sugar fraction. Further separation of the sugar fraction by chromatography revealed that, regardless of the great difference in the concentrations of nitrogen and phosphorus to which the seedlings were subjected during the experiment, their root systems contained the same soluble sugars: sucrose, glucose, fructose, raffinose, and very small amounts of some unknown soluble carbohydrates. As seen from table 1, the major soluble carbohydrate in the roots of all nutritional groups was sucrose; of the total ethanol-soluble C^{14} found in the sugar fraction, 53 to 91.4 percent was found in sucrose. In respect to glucose and fructose, it is of interest to note that at both phosphorus concentrations used by Björkman, i.e., 173 mg (P_1) and 692 mg (P_4) per liter, and nitrogen concentrations of 2.5 mg (N_0), 53 mg (N_1) and 265 mg (N_5) per liter had resulted in a definite increase in the amount of both sugars in the roots. The highest amounts of radioactive glucose and fructose were found to be present in those two nutrient groups in which moderate phosphorus concentration (173 mg/l) or the very high phosphorus concentration (692 mg/l) had been combined with the highest nitrogen concentration (265 mg/l). This finding is of particular interest, since these two nutrient groups in Björkman's experiment produced none or only few mycorrhizal roots (Björkman 1942, p. 134–137) and, in our experiments, entirely inhibited the formation of mycorrhizae.

Since all the data used in the discussion about sugar content in the roots were derived from the analysis of the whole root system, it may be questioned whether these data also apply to the sugar economy of the short roots, in which the actual symbiotic relationship becomes established. Meyer (1966, table 4), analyzing sugar content in different root regions of two-year-old *Fagus sylvatica* seedlings with abundantly formed mycorrhizae, revealed a definite sugar concentration gradient within the root system. The highest sugar content was found in the oldest, upper part of the tap root; the lowest, in the younger parts of tender roots infected by symbiotic fungi.

To obtain more exact information about the soluble sugar economy of short roots, in relation to different nutritional conditions, we recently analysed rootlets of white pine seedlings cultivated under three different nitrogen concentrations. The seedlings used for the experiment had grown for two years in a forest nursery. In the spring of the third growth season, they were excavated, potted in granitic sand, randomly divided into three nutrient groups, and grown under the same experimental conditions as described earlier (see p. 152). The seedlings received Björkman's (1942) basic nutrient solution with 173 mg/l of phosphorus and 2.5 mg, 53 mg, or 265 mg/l of nitrogen. In the fall, mycorrhizal roots collected from the two lower nitrogen level seedlings, and the nonmycorrhizal short roots collected from the highest nitrogen level seedlings, were analyzed by chromatography using a method described by Bidwell (1962). This method permits chromatography of crushed, fresh plant tissues placed directly on the filter paper. Chromatograms revealed that sucrose, glucose, and fructose were present not only in the well-developed mycorrhizal roots of the two lower nitrogen level seedlings but also in the nonmycorrhizal rootlets of seedlings grown under the highest nitrogen concentration. Even such a small quantity of short roots as 7 mg in fresh weight produced distinct spots of these sugars, and the amounts of individual sugars seemed to be practically the same in short roots of all nutrient groups despite the considerable differences in nitrogen concentration under which they had grown.

Ectomychorrhiza formation in relation to symbiotic fungus auxin

The complexity of ectomycorrhiza formation mechanisms is reflected by the characteristic morphology of these symbiotic organisms. Although ectomycorrhizae in nature may vary to a great extent, they have the following features in common: swellings, lack of root hairs on the swollen region, and radially-shaped cortical cells within the swellings. Melin (1925) convincingly demonstrated that these characteristic structures develop in the presence of the symbiotic fungus in his well-known mycorrhiza synthesis experiment *in vitro*. Recently, it was discovered that these profound morphological and anatomical changes in the short roots are induced by auxin-related metabolites which the symbiotic fungus exudes. It has been demonstrated experimentally that root structures strikingly similar to ectomycorrhizae can be synthesized in excised roots of *Pinus sylvestris* by adding to their nutrient solution a cell-free solution in which mycelium of a symbiotic fungus had grown for a certain period (Slankis 1948, see also Levisohn 1953 and Turner 1962). Similar morphogenesis can be induced by adding to the nutrient solution of Scots pine and white pine synthetic auxins in concentrations which are supra-optimal for roots (Slankis 1949, 1950, 1951, 1958, 1960). The ability of mycorrhiza-forming fungi to produce auxins in pure culture, if tryptophane is provided as precursor, has been reported by several investigators (Moser 1959, Ulrich 1960, Horak 1960).

Of importance to the principles involved in the formation of ectomycorrhizae is the discovery that these mycorrhiza-like morphological formations, synthesized either by fungous exudates or

with synthetic auxins, remained stable only as long as the cell-free exudates of the symbiotic fungus or the synthetic auxins were added periodically to the nutrient solution of the roots. A delay of the addition for more than two weeks resulted in an accelerated elongation of the swollen root apices, and gradually these synthesized mycorrhiza-like rootlets regained a nonmycorrhizal appearance (Slankis 1948, 1951). The newly-developed apical regions of such roots were not swollen but slender and densely covered with root hairs; the cortical cells within these regions had normal shape. Many of the synthesized simple and dichotomously branched mycorrhiza-like short roots, during their renewed elongation, developed into well-established long roots. This phenomenon indicated that, under the influence of the auxin, many potential long roots had been arrested at their primary growth.

The termination of mycorrhiza-like morphology when the addition of synthetic auxin was discontinued led to the speculation (Slankis 1958, 1959, 1961) that the structural characteristics of ectomycorrhizae actually reflect a specific physiological state in these roots and that the establishment of the symbiotic relationship is based on this physiological condition. Recently, experimental proof has been obtained for this assumption. It was found that well-established ectomycorrhizae also may undergo renewed elongation to such an extent that their characteristic morphology terminates and these roots acquire a nonmycorrhizal root structure and a nonsymbiotic state (Slankis 1967). Such a reverse morphogenesis of mycorrhizal roots can be demonstrated experimentally by subjecting seedlings with well-established mycorrhizae to nutritional conditions which inhibit symbiosis formation. For this experiment, two-year old forest nursery seedlings of white pine were used. They were carefully excavated in the spring, and only those with prevalent mycorrhizae were selected. The preparation of the seedlings for the experiment and the experimental conditions were again similar to those described earlier (see p. 152). The basic nutrient solution used in this experiment was the same as that used by Björkman (1942, p. 133). The four nutrient groups used contained phosphorus at 173 mg per liter, but the nitrogen concentration was varied so that each group received either 5 mg, 51 mg, 159 mg, or 265 mg nitrogen per liter.

Examination of the root systems at the end of the growth season revealed considerable differences in mycorrhizal development between seedlings grown under the two lower nitrogen concentrations and those grown at the two higher nitrogen levels (fig. 1). While at the two lower nitrogen levels, the majority of short roots, which developed during the experiment, had been converted into well-established mycorrhizae; the newly-formed short roots of the two higher level nitrogen seedlings had remained nonmycorrhizal. Of extreme interest was the finding that low and high nitrogen levels produced different effects in the well-established mycorrhizae, i.e., those mycorrhizal roots which had been formed in the nursery, prior to the experiment. At the two lower nitrogen concentrations these mycorrhizae retained their previous morphological features; at the two higher nitrogen concentrations their structures had changed considerably. The changes induced by high nitrogen concentration were strikingly similar to those which

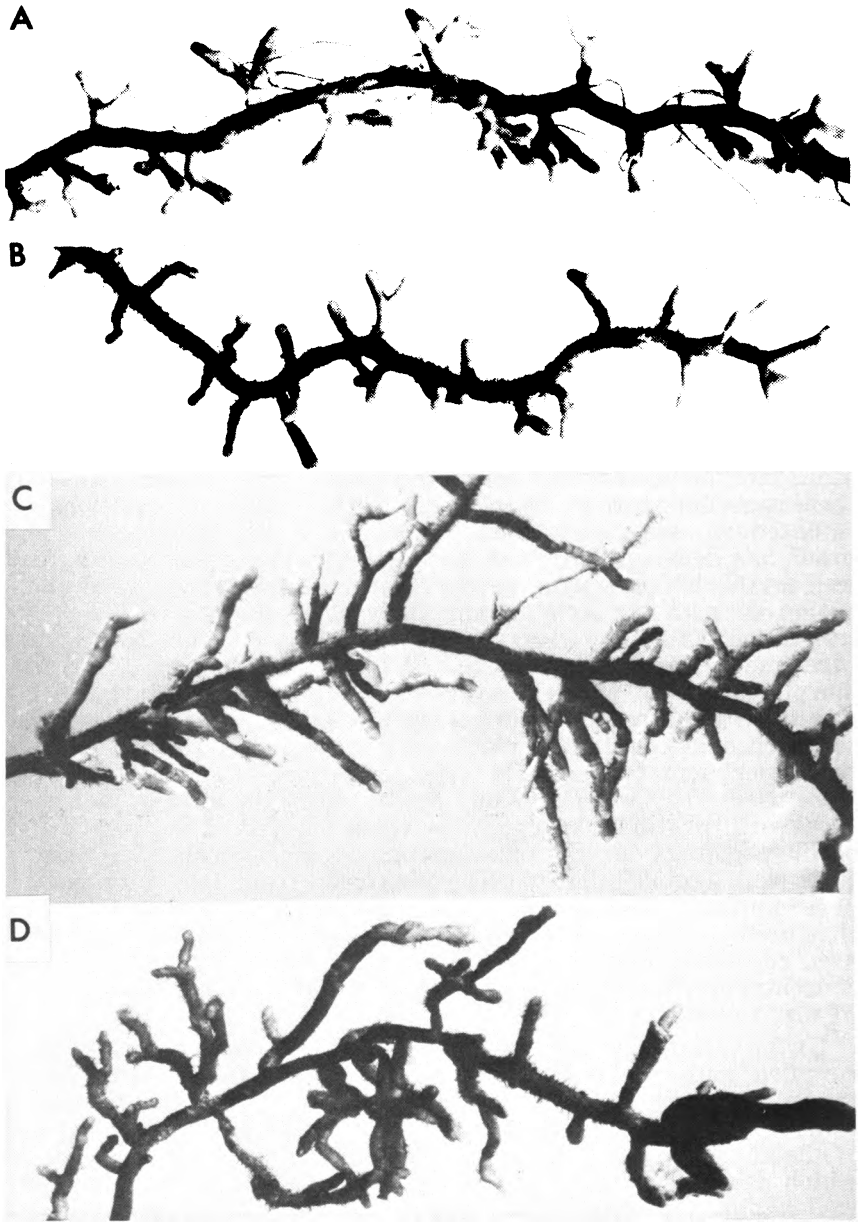

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Figure 1.—Root fragments of 4-year-old *Pinus strobus* L. seedlings grown under different nitrogen concentrations for the last 7 months. Fragment A (2.5 mg N/l) and fragment B (53 mg N/l) depict well-developed simple and dichotomous mycorrhizae, enveloped by a thick hyphal mantle. Fragment C (159 mg N/l) and fragment D (265 mg N/l) depict extensive renewed elongation of originally well developed mycorrhizal root apices as induced by an increase in nitrogen concentration. Note the dense root hair development on the newly formed apical regions. 4X.

synthesized mycorrhiza-like short roots underwent when periodic additions to the nutrient solution of either exudates of the symbiotic fungus or of the synthetic auxins were discontinued:

1. The swollen root apices of these ectomycorrhizae underwent extensive renewed elongation, and the newly formed root regions were slender and densely covered with root hairs;
2. The cortical cells in these elongated root apices had the same shape as in nonmycorrhizal roots;
3. Some of the extensively elongating apices of the simple and dichotomously branched mycorrhizae developed side roots, an indication that these mycorrhizae had become long roots.

Sections of mycorrhizal roots which had originated under nursery conditions revealed that different nitrogen concentrations had differently affected the intimate relationship between both symbionts. At lower nitrogen levels, these roots still exhibited well-developed Hartig nets and were covered with a hyphal mantle. At higher nitrogen levels, the cortical cells and Hartig net of the previously well-developed mycorrhizal region had been sloughed off, and, in the newly developed tissues, no traces of fungal hyphae were found.

The reverse morphogenesis induced in mycorrhizal roots at high nitrogen concentrations and the concurrent termination of the symbiotic relationship convincingly demonstrate that the characteristic morphology of ectomycorrhizae reflects a specific physiology of these roots. When this specific physiological state terminates, the symbiotic relationship also terminates.

Since synthesized mycorrhiza-like rootlets of pine undergo reverse morphogenesis when the periodic addition of the exudates of the symbiotic fungus or of synthetic auxin to the nutrient solution is discontinued, it appears that either high nitrogen concentrations inhibit production of the fungous auxin at levels necessary to maintain the specific physiological state in ectomycorrhizae, or that, under high nitrogen concentrations, the produced auxin is inactivated or degraded by the host plant roots (Siegel and Galston 1953, Andreae and Good 1955, Andreae and Ysselstein 1956).

Results derived from one of our more recent experiments suggest that, at high nitrogen concentrations, the synthesis of auxin by the fungal symbiont is inhibited. This experiment was carried out with white pine seedlings grown aseptically in specially designed culture tubes. The design of these culture tubes permitted aeration of the roots, periodic exchange of nutrient solution, and introduction of synthetic auxin. The growth room used for cultivation was air-conditioned. The light source was a combination of wide-spectrum "Grolux" and "Cool-daylight" flourescent tubes which produced a light intensity of 2500 ft-c. The length of photoperiod was 16 hours daily. The root systems of the seedlings were protected from light by insulating styrofoam covers which maintained a 19°C temperature at the root level. The temperature at the crown level was 25°C during the photoperiod and 19°C during darkness. The root systems of the seedlings were aerated daily at 45 minute intervals for a 15 minute period by means of an air pump and an interval timer. The nutrient solution, 500 ml per culture tube, was changed every second week. Before the experi-

ment was begun, the pine seedlings received a nutrient solution to which nitrogen (25 mg/l) in the form of ammonium nitrate was added. After 10 months of growth, the seedlings were randomly divided into two groups, designated as low-nitrogen and high-nitrogen seedlings, and the experiment began. During the experiment, the low-nitrogen seedlings received 5 mg nitrogen per liter, whereas the high-nitrogen seedlings received 159 mg nitrogen per liter in form of ammonium nitrate. As before, each culture tube contained 500 ml of nutrient and the nutrient was changed every second week. After 3 weeks growth under the different nitrogen levels, indoleacetic acid was added to the nutrient solution of half of the seedlings in each group. The other half of the seedlings remained as controls. The initial concentration of the auxin was 2.5 mg/l, but after the first three weeks, the concentration was increased to 5.0 mg/l.

Within two weeks, the presence of indoleacetic acid in the nutrient solution of both the low-nitrogen and the high-nitrogen seedlings had induced slight swellings in the old short root apices. Within three weeks, new short roots were initiated in large numbers. With the increase in auxin concentration their diameters gradually increased and these roots acquired swollen appearance. The swollen regions of the short roots lacked root hairs, and the cortical cells in these regions were radially shaped. However, under the influence of auxin, the root swellings of the high-nitrogen seedlings were considerably more pronounced than swellings of the low-nitrogen seedlings (fig. 2).

Ectomycorrhiza formation in relation to light

The role of light intensity in ectomycorrhiza formation also seems to be more complex than assumed earlier. Gast (1937), observing considerable reduction in mycorrhiza frequency of pine at low radiation intensities, concluded that this reduction is caused by a decrease in carbohydrate production. Björkman (1942) arrived at the same conclusion after growing pine seedlings under different light intensities. His findings demonstrate that every further decrease in light intensity below 25 percent of full daylight considerably decreases the soluble sugar content in the roots and, consequently, decreases mycorrhiza frequency. Björkman postulated that, along with the supply of available nitrogen and phosphorus in the soil, light has a paramount importance in controlling the formation of ectomycorrhizae. The importance of light for the formation of mycorrhizae has been confirmed by several investigators (Semikhnenko 1952, Wenger 1955, Harley and Waid 1955, Bouillard 1960, Shemakhanova 1962).

Recently, Handley and Sanders (1962) re-examined the influence of different light intensities in relation to readily soluble sugar content in the roots. Their experimental seedlings were established in the spring from *Pinus sylvestris* seeds and grown in sterilized quartz sand for 4½ months under light intensities of 50, 25, and 12 per cent of full daylight. The nutrient solution, with nitrogen 53 mg/l and phosphorus 173 mg/l, was the same as the one which in Björkman's experiment (1942) produced the highest frequency of mycorrhizae. At the beginning of November, the root systems of the experimental seedlings were analysed for the content of

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Figure 2.—Formation of swollen, mycorrhiza-like root structures induced by indoleacetic acid (IAA) (5 mg/l) in the roots of *Pinus strobus* L. seedlings at different nitrogen concentrations. Fragments: A—5 mg N/l, control; B—159 mg N/l, control; C—5 mg N/l and IAA; D—159 mg N/l and IAA. 4X.

soluble sugars. Despite the considerable differences in production of dry matter per seedling at different light intensities, there was no tendency for the amount of readily reducing substances in the roots to decrease with the decrease in light intensity. On the basis of these findings, Handley and Sanders disagree with Björkman's postulate and suggest a search be made for other factors which may be important in controlling formation of the symbiotic system in ectomycorrhizae. In their opinion, the increased concentration of easily soluable reducing substances occurring in roots of seedlings with prevalent mycorrhizae may not be the cause but the result of the established symbiotic association due to an accumulation of reducing substances in the fungal mycelium.

Our further studies on ectomycorrhiza morphogenesis lead to the conclusion that light may interact with the symbiotic fungus auxins in the formation of ectomycorrhizae. It seems that at low light intensity, the fungous auxin cannot induce the specific physiological and metabolic changes in the roots which are required for establishment of the symbiotic relationship. According to data reported by Björkman (1942), roots of Scots pine seedlings inoculated with mycorrhiza-forming fungi do not form mycorrhizae at light intensities 1/16 of full daylight. In our experiment (Slankis 1960), an addition of indoleacetic acid to the nutrients of white pine seedlings grown aseptically at a light intensity of 500 ft-c did not induce mycorrhiza-like swellings, whereas at 2500 ft-c, such swellings were readily induced (fig. 3).

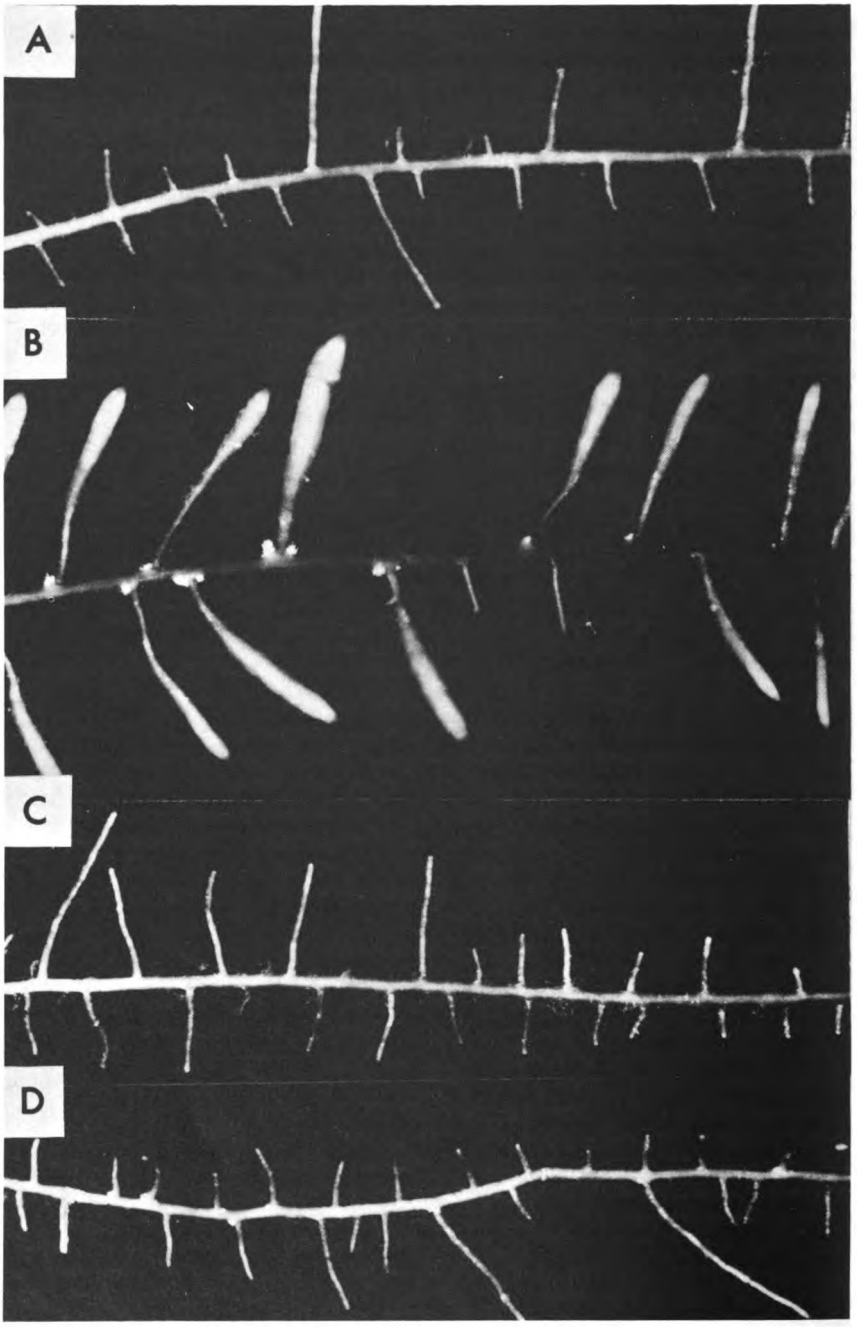

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Figure 3.—Formation of mycorrhiza-like swellings on *Pinus strobus* short root apices induced by IAA under different light intensities. Fragments: A—2500 ft-c, control; B—2500 ft-c and IAA; C—500 ft-c and IAA; D—500 ft-c, IAA and glucose 5 g/l. Auxin concentration during 1½ month period gradually increased from 0.1 mg/l to 3.2 mg/l. 3.5X.

Since an addition of glucose, fructose, or sucrose to the nutrient solution at low light intensity did not initiate any change, it indicates that the failure of the fungous auxin to induce the specific physiological state at low light intensity can hardly be ascribed to a possible deficiency of soluble sugars in the roots. More likely, the formation of this specific physiological condition results from an interaction between the fungous auxin and some host plant metabolites other than sugars. It seems that these metabolites, which formed a low light intensity, are inhibitory to auxin. It is of interest to note that long roots of the control seedlings at low light intensity continued to elongate and produce side roots during the experiment. This indicates that under low light intensity, photoassimilation still continues and that formed assimilates are translocated to roots.

The importance of stem metabolites in the formation of the symbiotic relationship becomes apparent from the report by Fortin (1966). Experimenting with excised roots of *Pinus sylvestris,* he was unable to sustain their continuous growth without leaving a 5 mm piece of hypocotyl attached. By supplying the hypocotyl with sucrose, thiamin, and choline chloride and the roots with inorganic nutrients, he succeeded in obtaining well-developed mycorrhizae after the roots of the explants were inoculated with a proper symbiotic fungus.

Conclusion

In summary, it it apparent that the establishment of ectomycorrhiza is a complex process in which several factors interact.

Although generally it is assumed that inorganic fertilizers are inhibitory to mycorrhiza formation, several recently published reports show that, in some cases, a large dosage of nitrogen and phosphorus may enhance this formation (Eglite 1955, Meyer 1962, 1963, Shcherbakov and Mishustin 1950, Pushkinskaya 1952). Even in nutritionally rich soils, an introduction of additional nitrogen and phosphorus may bring about an increase in mycorrhiza frequency (Meyer 1962, 1966). It may be that the formation of the symbiotic association in short roots is controlled, not so much by the actual concentrations of nitrogen and phosphorus, but by the ratio between these two elements, as suggested by Shemakhanova (1962).

Contrary to an earlier view (Björkman 1942), it has become apparent that an excess of soluble sugars may accumulate in the roots of pine grown at relatively high nitrogen and phosphorus concentration (Meyer 1962, 1966, Slankis 1965, Lister *et al.* 1968). Table 1 shows that if the phosphorus concentration is held constant while the nitrogen concentration is increased from 2.5 mg to 265 mg per liter, there is a slight decrease in the accumulation of the major sugar, sucrose, but that the concentrations of glucose and fructose increase. Thus, the greatest accumulation of these two sugars was found in roots of pine grown in the nutrients which combined the highest concentrations of nitrogen and phosphorus. As mentioned, Handley and Sanders (1962), referring to Björkman's (1942) data on soluble sugar content in roots, suggested that a certain amount of the increased soluble sugar content found by Björkman in mycorrhizal roots might have been derived from the hyphae of the

fungal associate. This possibility is inconsistent with our findings since under those nutrient regimes in which the accumulation of glucose and fructose in pine roots was the highest, mycorrhiza formation was inhibited.

Meyer (l.c.), who found that an increase in nitrogen and phosphorus concentration resulted in the increase both of the soluble sugar content in the roots and of mycorrhiza frequency, concluded that a surplus of soluble sugars in the roots is not the cause of mycorrhiza formation but that the accumulation of these sugars in the roots is caused by the symbiotic fungus-auxins. He based this conclusion on reports by Borthwick *et al.* (1937), Stuart (1938), Alexander (1938) and Bausor (1942) that an addition of auxin to plants enhances the hydrolysis of starch into sugars. Furthermore, in the light of findings by Cleland (1961), Meyer assumed that the fungous auxin also affects the transport of soluble sugars from those root regions where the reserve starch is stored to those locations where the auxin is secreted, i.e., to the mycorrhizal short roots. In fact, it has been found that synthetic auxin supplied externally to one region of the root system of pine seedlings shows its effect throughout the whole system (Slankis, unpublished data). However, the results reported by Lister *et al.* (1968) reveal that an increase in soluble sugar content in the roots may occur also at those high nitrogen and phosphorus concentrations which entirely inhibit formation of the symbiotic relationship.

The assumption that soluble carbohydrates are the sole factor in the formation of ectomycorrhiza is not tenable in all instances. Evidently the presence of a surplus of soluble sugars in the roots does not always bring about an ultimate formation of ectomycorrhiza. Even a relatively high sugar content in the root system (Lister *et al.* 1968) and in short roots (Slankis 1965) may fail to produce mycorrhizae if these sugars accumulate when pine seedlings are grown under high nitrogen and phosphorus concentrations (table 1). That the presence of soluble sugars alone in the roots does not assure mycorrhiza formation is most convincingly demonstrated by the phenomenon that well-established mycorrhizal roots regain a nonmycorrhizal state when nitrogen and phosphorus concentrations in the nutrients are increased (fig. 1). However, as seen from table 1, this reverse process is not caused by a decrease or a deficiency in soluble sugars in the roots. So it appears, that although the host plant sugars clearly are significant in the growth and development of the symbiotic fungus (Melin, 1953), the role of these sugars in the formation mechanism of the symbiosis can be questioned.

Although a correlation between light intensity and mycorrhiza frequency has been confirmed by several investigators (Gast 1937, Semikhenko 1952, Wenger 1955, Harley and Waid 1955, Hacskaylo and Snow 1959, Boullard 1960), the mode of action of light in the mechanism of mycorrhiza formation is also still not clear and needs further study. The opinion expressed by Björkman (1942), that the combination of light intensity and the concentration of available nitrogen and phosphorus regulate soluble sugar content in the roots and hence the formation of ectomycorrhizae, is challenged by findings of Handley and Sanders (1962). They have demonstrated experimentally that when light intensity is decreased from 50 to

12 percent of full daylight, there is no tendency for the amount of soluble sugars in pine roots to decrease. As already mentioned, these authors suggest that we seek other factors which may control the formation of the symbiotic relationship.

The finding that mycorrhiza-like swellings are not formed in pine roots by synthetic auxins at low light intensities, even when soluble sugars are added to their nutrient solution (Slankis 1960), indicates that light intensity affects the fungous auxin activity in some yet unknown way and thus the profound physiological changes in short roots required for mycorrhiza formation fail to be induced. It seems that the establishment of the specific physiological state results from an interaction between the fungous auxin and the host plant metabolites, other than soluble sugars and that certain of these metabolites are not formed at low light intensities. Also results obtained by Fortin (1966) with pine explants suggest that stem metabolites play an important role in mycorrhiza formation, since in his experiment excised roots of *Pinus sylvestris* inoculated with a mycorrhiza-forming fungus produced well established mycorrhizae only when a 5 mm portion of the hypocotyl remained attached to the radicle and organic nutrient was supplied only to the remnant of the stem. The mycorrhizae of his explants had a Hartignet while the intracellular invasion by hyphae was limited. Although *Pinus sylvestris* roots excised below the hypocotyl also produce mycorrhizal structures in the presence of a symbiotic fungus (Slankis 1948), the intracellular invasion by the fungal hyphae is very extensive (Melin and Slankis, unpublished data).

The characteristic morphology of ectomycorrhizae is not, as previously thought (see Melin 1953, Harley 1959), just a structural deviation from nonmycorrhizal roots caused by the fungus-auxin. Sufficient evidence has accumulated to support the view (Slankis 1958, 1961) that this structural deviation is caused by the profound physiological and metabolic changes which the nonmycorrhizal short roots undergo during their conversion into ectomycorrhizae. It appears then that this symbiotic relationship is actually based on a specific physiological state which results from the interaction between the fungous auxin and the host plant metabolites. The specific physiology in mycorrhizal roots seems to function as a pathway through which the fungal symbiont is able to obtain sugars and other vital metabolites from the roots. Furthermore, it has become apparent that in order to establish and maintain this specific physiology in mycorrhizal roots, the fungal symbiont must be able to produce and transfer into these roots a more or less continuous supply of auxins at supra-optimal concentrations throughout the growth season. If the auxin production becomes inhibited, the specific physiological state terminates and with it, the symbiotic relationship. As a result of these two concurrent processes, the mycorrhizal roots undergo extensive elongation and regain a nonmycorrhizal state and a nonmycorrhizal morphology (fig. 1).

Of further interest is that synthetic auxin induces more pronounced ectomycorrhiza-like swellings in pine roots at high nitrogen concentrations than at low concentrations (fig. 2). This phenomenon suggests that a high nitrogen concentration does not

increase the ability of the host plant to inactivate or destroy auxin derived from an external source. On the contrary, under such nutritional conditions the effect of auxin increases. Logically, we may presume that the effect of the fungous auxin in fertile soils would be similarly enhanced if, under such nutritional conditions, the fungus would produce auxins. Since at high nitrogen concentrations, the characteristic ectomycorrhizal morphogenesis is not induced and the symbiotic relationship does not establish between both symbionts, one is lead to conclude that high nitrogen concentrations either prevent the fungous auxin production or inhibit the symbiotic fungus from producing auxin in sufficient concentrations. These possibilities are indicated by the fact that the reverse morphogenesis in ectomycorrhizae induced by an increase in nitrogen concentration is strikingly similar to that which synthesized mycorrhiza-like short roots undergo when the periodic supply of synthetic auxin is discontinued (Slankis 1951). In both cases, these roots regain nonmycorrhizal morphology; in the case of ectomycorrhizae, they also regain a nonmycorrhizal state.

We may presume that this inhibited auxin production by the symbiotic fungus at high nitrogen concentration is caused by root metabolites and root exudates, the composition of which changes with a change in nitrogen concentration (fig. 4). The soil nitrogen may also exert an inhibitory effect directly upon the fungus hyphal network. According to a report by Moser (1959), the presence of certain amino acids in the nutrient solution of a symbiotic fungus in pure culture had considerably inhibited the production of fungous auxin. That some amino acids exert strong stimulatory or inhibitory effects on mycelial growth of symbiotic fungi becomes apparent from data published by several investigators (see Melin 1953). Chromatograms, obtained by Bidwell's (1962) method, of amino acids from short roots of white pine seedlings grown under

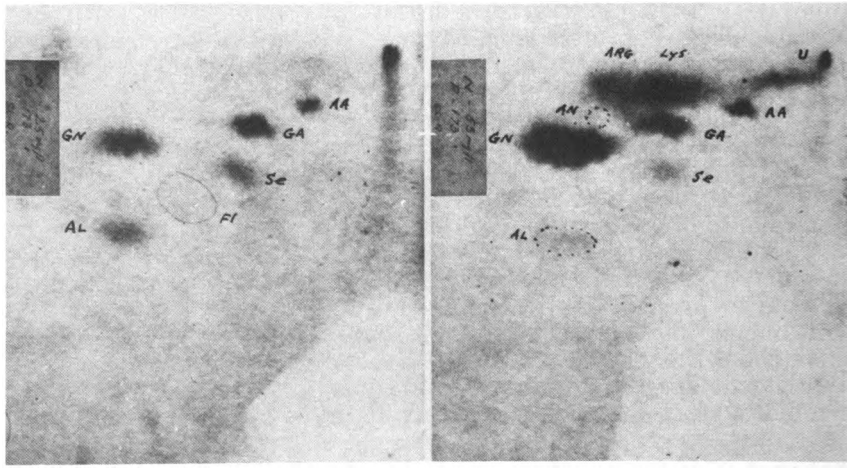

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Figure 4.—Two-dimensional paper chromatograms of free amino acids present in short roots of *Pinus strobus* L. seedlings grown under nitorgen concentrations 2.5 mg/l (left) and 53 mg/l (right): AA, aspartic acid; AL, alanine; AN, asparagine; ARG, arginine; GA, glutamic acid; GN, glutamine; LYS, lysine; Se, serine; U, unknown; Fl, fluorescent spot.

different nitrogen concentrations reveal that with an increase in nitrogen concentration, there is an increase in the number of amino acids present and also a considerable increase in the amount of some of these amino acids (fig. 4).

Since 1923, Melin has repeatedly expressed the opinion that the symbiotic relationship in ectomycorrhizae is based on the principle of controlled parasitism. In Melin's opinion, the symbiotic fungus is an aggressor with a tendency to gain a maximum nutritional benefit from the host plant, but these parasitic tendencies are restricted by the higher plant to a degree that ensures mutual symbiosis. In my experiments, the induced ultimate cessation of well-established symbiotic relationships in ectomycorrhizae of pine, when nitrogen is provided in excess, clearly demonstrates the ability of the higher symbiont to control its fungal partner. Evidently, the symbiotic fungus is able to maintain the established intercellular contact with the host plant root tissues only at nutritional conditions that are sub-optimal for the higher symbiont, i.e., under conditions at which the host plant cannot obtain sufficient nutrient without the aid of the fungus. However, when the nutritional conditions improve, the previously mycorrhizal roots regain a nonmycorrhizal state and this intimate relationship for the fungus in the roots terminates.

Little is known about the mechanism in the host plant which controls the entrance of fungal hyphae into the root tissues. Melin (1959, 1963) found that roots produce both a growth stimulant factor (M-factor) and a growth inhibiting factor of fungicidal nature. He suggested that these factors largely control the activity of the symbiotic fungus. It now appears that the formation of ectomycorrhiza is a complex process far from being fully understood. The establishment of this symbiotic relationship requires that the fungus not only gain entrance into the host plant roots, but also induce and maintain a specific physiological state in these roots. This seems to be a very involved process in which the fungus-auxin interacts with certain host plant metabolites. However, the formation of the fungous auxin and of the host plant metabolites are themselves controlled by several environmental factors. Of these factors, light and nutrients particularly play an important role.

Acknowledgment

The author wishes to express his sincere thanks to Miss Jean F. Robinson for taking the photographs and preparing the illustrations.

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Separate No. FS-294

14.

Cytokinin Production by Mycorrhizal Fungi

Carlos O. Miller

Cytokinins perhaps are best defined by the array of growth and developmental responses they elicit in plants or plant parts. Under particular conditions, they promote cell division, modify cell enlargement, promote new shoot formation, favor outgrowth of lateral buds ordinarily suppressed by apical buds, inhibit root elongation, decrease response to auxin by stem segments, and promote seed germination. They also influence synthesis of phenolic compounds and related pigments, help to maintain chlorophyll, protein and ribonucleic acid levels in detached plant parts such as leaves, mobilize various materials to the point of cytokinin application, increase stomatal opening and therefore transpiration, apparently protect against certain deleterious effects of both high and low temperatures and of wilting, and help to protect against invading organisms (reviews: Miller, 1961; Letham, 1967a; Helgeson, 1968).

The compounds most effective as cytokinins are adenine derivatives in which the six-amino group has been modified by the replacement of a hydrogen atom with some fat-soluble group; many different substituents work. Kinetin (6–furfurylaminopurine) is an example of this type of cytokinin (fig. 1). A less effective type

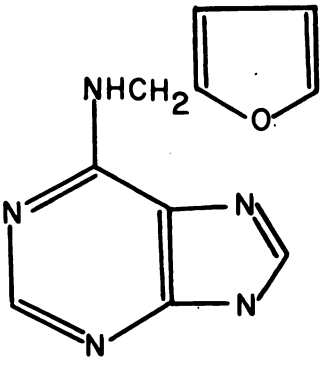

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Figure 1.—The structural formula of kinetin.

of compound is represented by *sym*-diphenylurea; many related compounds also have activity (see Bruce, Zwar and Kefford, 1965). Some active adenine derivatives are now known to occur naturally, but there is doubt that the substituted ureas do.

Our interest in the possibility that mycorrhizal fungi may produce and release cytokinins sprang from the facts that, in mycorrhizae, a common growth modification is the enlargement of root cortex cells and that this modification may also result from treatment with kinetin (Arora, Skoog and Allen, 1959, for example).

Methods and Results

Some assays for cytokinins currently in use include cell division tests with soybean, tobacco or carrot tissues, enlargement of leaf or cotyledon pieces, chlorophyll retention in detached leaves, bud formation in moss protonemata (Hahn and Bopp, 1968), germination of lettuce seeds, formation of compounds such as betacyanine (Conrad and Köhler, 1967), and transpiration by detached leaf blades (Luke and Freeman, 1967). Information concerning bioassays may be obtained from discussions by Miller (1963 and 1967c) and Letham (1967b). In our work with fungi, we have used only the soybean callus tissue assay. This tissue is kept in continuous culture and is derived from a piece of cotyledon of *Glycine max* L. Merrill, ev. Acme, put into culture in 1959. Since that time, the tissue has been subcultured on a synthetic medium. This medium contains (mg/1): KH_2PO_4, 300; KNO_3, 1000; NH_4NO_3, 1000; $Ca(NO_3)_2 \cdot 4H_2O$, 500; $MgSO_4 \cdot 7H_2O$, 71.5; KCl, 65; $MnSO_4 \cdot 4H_2O$, 14; NaFe ethylenediaminetetraacetate, 13.2; $ZnSO_4 \cdot 7H_2O$, 3.8; H_3BO_3, 1.6; KI, 0.8; $Cu(NO_3)_2 \cdot 3H_2O$, 0.35; $(NH_4)_6\ Mo_7O_{24} \cdot 4H_2O$, 0.1; *myo*-inositol, 100; nicotinic acid, 0.5; pyridoxine · HCl, 0.2; thiamin · HCl, 0.1; ∝-napthalene-acetic acid, 2; sucrose, 30,000; kinetin, 1; and Bacto-agar, 10,000. The pH is adjusted to 5.8. For assay, small pieces (about 4 mg each) from lumps of tissue about 5 weeks old are transferred to this same medium but with the kinetin omitted.

In checking for cytokinin production by the fungus, several approaches may be used. In the most simple and direct procedure, an inoculum of the fungus is placed on the agar surface near the soybean tissue pieces. These tissue pieces will grow only if the fungus releases a cytokinin. An example of a positive result is presented in figure 2. The fungus pictured here is *Rhizopogon roseolus*. The basal medium without kinetin added was used in the flask in which the soybean pieces have not grown. This same medium was used in the flask in which the fungus was also present. The third flask is a positive control with 1 mg/l kinetin added. Apparently, the fungus and kinetin in some way do the same thing. That the action of the fungus probably is dependent upon the diffusion of a substance from the fungus is indicated in figure 3. A gradient in growth response is evident in pieces of soybean tissue placed at different distances from the inoculum of *R. roseolus*. Results of this type led us to believe that the fungus indeed produced and released one or more cytokinins. Assays of liquid culture media in which the fungus had grown confirmed this. Eventually, from about 200 liters of such liquid media, we isolated in crystalline form about 1 mg each of the two most abundant cytokinins. These two were identified as zeatin (fig. 4) and the ribonucleoside of zeatin. A third but minor cytokinin probably was zeatin ribonucleotide (Miller, 1967a). All of these compounds had earlier been obtained from maize kernels (Letham, Shannon and McDonald, 1967; Miller, 1967b). Zeatin and derivatives also are known to occur in plums and sunflowers (Klämbt, 1968) and there is fairly good evidence of their existence in many other species of higher plants. Using this approach for detection, cytokinin production by three other species of fungi has been established. The species are: *Suillus cothurnatus, S. punctipes,*

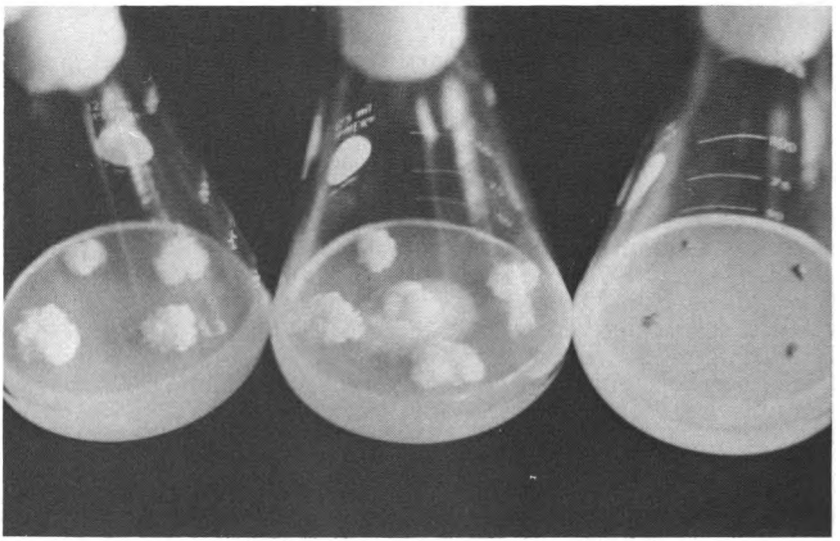

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Figure 2.—The apparent production of cytokinin by the fungus *Rhizopogon roseolus*. Soybean tissue pieces were planted on the agar surface in all three flasks, and all flasks contained the basal medium. The flask on the left, however, also contained 1 mg/l kinetin. In the center flask, an inoculum of the fungus was placed in the center of the four soybean pieces.

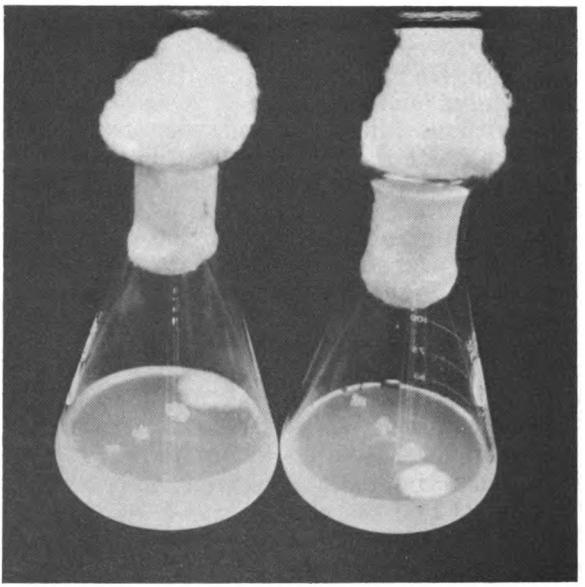

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Figure 3.—Diffusion of cytokinins from the fungus *Rhizopogon roseolus*. The two flasks were replicates and contained the basal medium without any cytokinin. The fungus was placed at the end of a row of soybean tissue pieces. (Demonstration by T. S. Moore, Jr.)

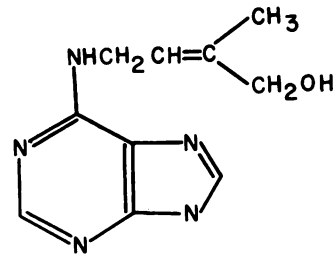

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Figure 4.—The structural formula of zeatin.

and an unidentified ectendomycorrhizal species (#57 from the U. S. Department of Agriculture) (fig. 5).

The culture of a fungus and soybean tissue on a common gel may not always be valid as a check for cytokinin production. The fungus may grow too fast and overgrow the tissue; it may produce toxic materials which inhibit soybean growth, or the fungus may not produce enough cytokinins to be detected even though the soybean assay responds to zeatin at a concentration as low as 5×10^{-11} M. (Incidentally, we have also noticed that, in some instances, the soybean tissue inhibits the growth of the fungus.) If with this test negative or indefinite results are obtained with a particular fungus, we then grow that species in a liquid medium and examine the medium filtrate in a variety of ways. The filtrate may be concentrated for further testing. One may attempt to separate the cytokinins from inhibitory substances by paper chromatography, column fractionation (Dowex 1 works for purine ribonucleotides, Dowex 50, for free bases and ribonucleosides), or by solvent extrac-

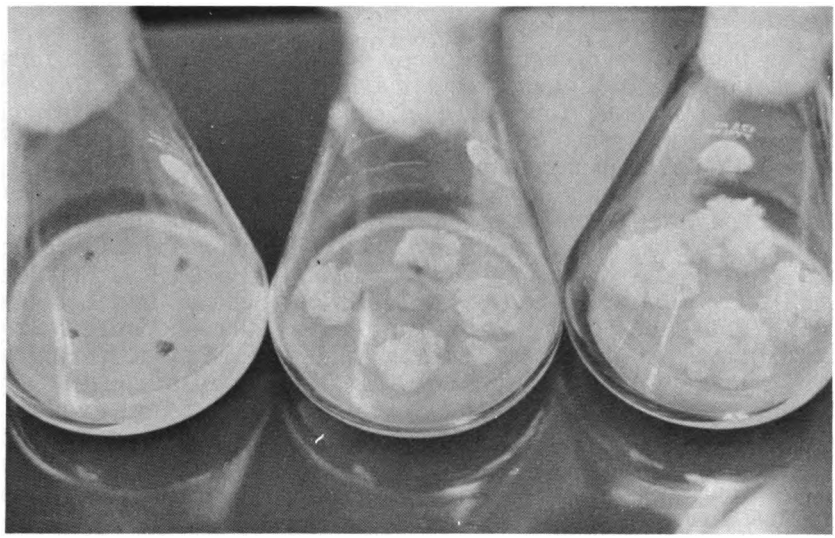

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Figure 5.—The apparent production of cytokinins by an unidentified ectendomycorrhizal fungus species. All flasks contained the basal medium and the flask on the right also contained 1 mg/l kinetin. In the center flask, an inoculum of the fungus was placed in the center of the four soybean tissue pieces.

tion. Fractions or eluates are assayed with soybean tissue by incorporating them into the basal medium without kinetin. Using some of these techniques, we have detected cytokinin production by *Amanita rubescens* (Miller, 1967a). Using all of these methods, we have been unsuccessful in detecting activity from *Cenococcum graniforme* and *Thelephora terrestris*. The fungi mentioned thus far are known to form mycorrhizal associations. Results indicate definite cytokinin production by five and probably no production by two of the mycorrhizal fungi. We hope to obtain more species to test.

Scleroderma geaster, which has not been proven to be mycorrhizal, apparently does not produce cytokinins, but does release substantial amounts of auxins. This is of interest because auxins and cytokinins show an extremely large number of interactions in plant growth phenomena. (The production of auxin by *S. geaster* was detected by using the basal medium with the kinetin added but with the naphthalene acetic acid omitted. This works since cell division in the soybean tissue is dependent upon exogenous supplies of both an auxin and a cytokinin.)

Several fungi which probably are not mycorrhizal have been examined for cytokinin production. No clearly positive results have been obtained with any of them. They are: *Armillaria mellea, Collybia velutipes, Coniophora suffocata, Coprinus cornatus, Cyathus stercoreus, Fomes connatus, F. everhartii, F. johnsonianus, F. pinicola, F. pomaceous, Morchella angusticeps, M. crassipes, M. esculenta, M. rotunda, Phallus impudicus, Polyporous palustris (A* and A1), *Schizophyllum commune, Stereum hirsutum, S. purpureum,* and *S. striatus.*

Discussion

The production of cytokinins by bacteria or fungi that form associations with higher plants may be quite common. *Corynebacterium fascians,* for example, causes host plants to branch excessively, apparently by producing large amounts of 6-dimethylallylaminopurine, a compound which has good cytokinin activity (Klämbt, Thies and Skoog, 1966; Helgeson and Leonard, 1966). Extracts from *Uromyces phaseoli* and *U. fabae* uredospores (Király, Pozsár and El Hammady, 1966), *Puccinia graminis* tritici uredospores and *Erysiphe graminis* spores (Bushnell and Allen, 1962), *P. recondita* uredospores (Johnson, Schafer and Leopold, 1966) and from galls of *Brassica rapa* L. roots infected by *Plasmodiophora brassicae* Woronin (Matsubara and Nakahira, 1967) give cytokinin effects. Any parasitic organism causing any of the previously mentioned cytokinin effects on higher plants might logically be examined for cytokinin production.

What advantages might occur because of cytokinin production by microorganisms? One possibility is that the cytokinins keep the host tissue in a healthy condition and therefore better able to support the parasite, for example, the green island effect observed with rusts (Pozsár and Király, 1964). The maintenance of chlorophyll levels by cytokinins is well established, and green cells usually are nutritionally healthy cells. In that the cytokinins apparently cause accumulation or mobilization of various materials, both organic and inorganic, in regions to which they are applied, cytokinin

production by the parasitic organism may cause more nutrients to become available to it. Another possibility, not very well explored yet, is that the cytokinins actually aid the host in limiting the spread or even takeover by invading organisms. In this connection, cytokinins are known to stimulate production of several phenolic types of compounds in higher plants (Miller, 1969). Such phenolic compounds may aid the host in slowing parasite growth. We are now spending much time in a study of the production of the deoxyisoflavone, daidzein, and related compounds. Such production is very sensitive to the presence of cytokinins.

There is another aspect to consider when thinking of the mycorrhizal relationship. The root tip may be a main site of cytokinin production in the higher plant (Weiss and Vaadia, 1965). If an invading mycorrhizal fungus shuts off such production, the host plant might suffer. Perhaps the fungus takes over the job of cytokinin synthesis. In doing so, it would expose cortex cells to cytokinins, causing enlargement of the cells as well as loosening of the tissue and would cause accumulation of nutrients in the region of fungal growth. Cytokinin production could aid both the host and the fungus.

Summary

Seven mycorrhizal fungi have been examined for the release of cytokinins. Five of the fungi do release such compounds, but the other two apparently do not. Twenty-two fungi, which are not known to be mycorrhizal, apparently do not give off cytokinins.

Acknowledgments

A considerable portion of the preliminary screening of fungi for cytokinin production was done by Mr. T. S. Moore, Jr. Many of the fungi were supplied by Dr. E. Hacskaylo of the U. S. Department of Agriculture. The project was supported by grant GE3612 from the National Science Foundation.

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Separate No. FS–295

15.

Metabolite Exchanges in Ectomycorrhizae

Edward Hacskaylo

It is becomingly increasingly evident that nutrient absorption through mycorrhizal systems and metabolite exchanges between the symbionts are dependent upon a multiplicity of interactions. Ten years ago, it seemed possible to synthesize a reasonable general account of the physiological processes regulating mycorrhizal formation and to define many of the interactions between host and fungus. Today, several theories, particularly on the initiation of mycorrhizal infections, are being challenged. The advent of sophisticated techniques, including use of radioactive isotopes, and the increase of competent researchers in the field make it imperative that data of earlier workers be constantly reappraised to determine whether their conclusions remain valid or whether reinterpretations are in order. This paper includes an examination of some of the theories on metabolic interactions in ectomycorrhizae as they relate to each other and some interpretations based on data from our laboratory and from experiments of others.

History

Between 1885 and 1950, most research on ectomycorrhizae was directed toward clarifying the concepts proposed by Frank (1885) and Stahl (1900). Frank was convinced that a high proportion of the absorbing roots of forest trees were invaded by mycorrhizal fungi and suggested that mycorrhizae were a symbiotic relationship. Stahl proposed that the mycorrhizal fungi were more efficient than root hairs in absorption of inorganic nitrogen and that mycotrophy was essential for the host in soils low in nitrogen availability. Numerous descriptive papers by other authors contain information showing that mycorrhizae indeed were even more cosmopolitan than Frank had suggested. Melin (1925) broadened Stahl's concept by theorizing that mycorrhizal fungi not only were efficient in inorganic nitrogen absorption for the host, but also could be important in the uptake of organic nitrogen, potassium, and phosphorus. He also observed that metabolites released by pine roots stimulated growth of mycorrhizal fungi. Melin referred to the root metabolites as phosphatides, compounds that were active in very small quantities.

Hatch (1937) studied the responses of pines to mycorrhizal infections in the field and in the laboratory, particularly in relation to nutrition. He concluded that the mycotrophic relationship in ectomycorrhizae is a symbiotic mechanism which increases, chiefly by physical, relatively nonselective means, the absorption of soil nutrients. The greater absorption capacity was causd by increases in the effective absorbing surface areas of short roots resulting from fungal invasion. He suggested that the benefits to the mycor-

rhizal fungi probably were in the nature of growth-promoting substances originating within the roots. He also theorized that susceptibility to infection by mycorrhizal fungi is controlled indirectly by the internal concentration of nutrient elements in short roots. Results of experiments with seedlings in nurseries (Mitchell, Finn, and Rosendahl 1937; Routein and Dawson 1943; McComb 1938, 1943) left little doubt that ectomycorrhizal associations resulted in seedlings that were thriftier than nonmycorrhizal ones.

Björkman (1942) attempted to define the controlling mechanism in mycorrhizal infection and concluded that an excess of soluble carbohydrates in short roots is essential to induce fungus penetration. By reducing light intensity, or by physically interrupting the translocation of carbohydrates from shoots to roots, mycorrhizal formation decreased or ceased. Björkman also suggested that increased availability of nitrogen and phosphorus in the growing medium resulted in greater utilization of photosynthate in the shoot, and hence, the amount of carbohydrate translocated to the roots was reduced. This, he believed, was the reason for suppression in mycorrhizal formation under such conditions.

Hacskaylo and Snow (1959) confirmed that formation of mycorrhizae on pine was suppressed by high levels of available nutrients and low light intensities. They favored the hypothesis produced by Björkman but stated that "conditions that permit and promote formation of mycorrhizae no doubt are controlled by a complex of both internal and environmental factors. These conditions are only partially known and deserve more study."

Handley and Sanders (1962) thereafter reported results of a series of experiments similar to Björkman's on the effects of nutrients and light intensity on formation of mycorrhizae. They concluded that concentrations of soluble reducing substances increased as a result of rather than as a precursor to mycorrhizal associations. They suggested that accumulations of reducing substances occurred in the fungal mycelium. Meyer (1962) also attributed increases in sugar concentration in mycorrhizae to the associated fungi and to an active microbial population in the soil.

Slankis (1951) reported that the cause of the morphological changes in ectomycorrhizae was fungus auxin. Synthetic growth regulators such as indoleacetic acid, indolebutyric acid, and napthalene acetic acid produced morphological changes in pine short roots remarkably similar to those that are found when the shoot roots become mycorrhizal. Later Slankis (1961) expressed reservations about Björkman's carbohydrate theory and concluded that establishment of ectomycorrhizae is a very complicated physiological mechanism not fully recognized in previous theories.

Melin (1954) discovered that at least one metabolite, which he called the "M-factor," produced by roots, greatly stimulated the growth of mycorrhizal fungi. His subsequent experiments illustrated dramatic increases in growth of the mycelia of mycorrhizal fungi in the presence of the chemically undefined "M-factor." Melin (1955) also obtained data showing that an antifungal substance that helped maintain a balanced parasitic relationship between the host and associated fungus was produced within roots.

As techniques involving use of radioactive isotopes became available to plant scientists, studies on uptake of mineral nutrients

through mycorrhizal fungi progressed rapidly. After 50 years of circumstantial evidence, Kramer and Wilbur (1949) demonstrated accumulation of radioactive phosphorus in pine mycorrhizae. Melin and Nilsson (1950) showed conclusively that hyphae of mycorrhizal fungi could translocate nutrients from the substrate to the host in greater quantities than nonmycorrhizal roots. Harley and his associates (see Harley 1959) have intensively studied uptake and translocation of nutrients in beech mycorrhizae. Their data, accumulated primarily with excised mycorrhizae, show that the fungus sheath absorbs phosphate ions actively and accumulates phosphorus in greater quantity than the host tissue. Phosphate is stored and released slowly but steadily to the host. In periods of phosphate efficiency followed by periods of phosphate deficiency, Morrison (1957) found that phosphate ions moved steadily to the shoots of mycorrhizal *Pinus radiata* plants, but little translocation occurred in nonmycorrhizal seedlings after the first few days. On the other hand, Morrison (1962, 1963) found that sulfur did not accumulate in mycorrhizae but passed freely through the mycelium of the mycorrhizal fungus. Carrodus (1966) reported that beech mycorrhizae are capable of absorbing ammonia and simple organic compounds from solution, but nitrate is a poor source of nitrogen and may not be absorbed at all. By supplying an exogenous source of glucose or fructose, which caused an increase in metabolic rates, the rate of ammonia absorption increased. Melin and Nilsson (1957), attempting to prove that reciprocal exchanges of metabolites occurred between fungus and host, exposed needles of aseptically grown pine seedlings to C^{14} and traced its movement through the plants into the attached hyphae of their mycorrhizae. Björkman (1960) thereafter showed that movement of radioactive carbon and phosphate from mature spruce trees to *Monotropa* plants can occur via common mycorrhizal fungus hyphae.

Interpretations

Early Research

On the basis of the above reports and others, a general summary of the metabolic interactions in ectomycorrhizal associations would read as follows: Initial contact between hyphae of a mycorrhizal fungus and a compatible short root may originate from spores germinated in the vicinity of the roots, by extension through the soil of hyphae from either residual mycelia or established mycorrhizae, or by progression of hyphae through adjacent internal root tissue. Thereafter many interactions are initiated between the fungi and the root cells.

Growth of mycorrhizal fungi on the surfaces of the roots is stimulated by exudates from the roots. These exudates contain at least one growth-promoting metabolite designated as the M-factor. Dependency on the M-factor varies widely with the various species of mycorrhizal fungi.

Entrance of ectomycorrhizal fungi into the roots requires secretion of pectolytic enzymes which dissolve the middle lamella and thus permit the hyphae to grow through the intercellular regions of the cortex. Physical or chemical properties of the roots or perhaps both, restrict the hyphae of all mycorrhizal fungi to the cortex

delimited by the endodermis and meristematic cells of the root tip. The exact mechanism of this resistance to the hyphae is unknown.

The growing, absorbing roots of several species of forest trees which have become enveloped by ectomycorrhizal fungi are subjected to a growth regulatory substance produced by the fungi. This fungus auxin modifies subsequent root growth, retards elongation of short roots, and frequently initiates dichotomous branching in pine. The cortex cells are oriented somewhat differently from those in nonmycorrhizal roots, and presence of intercellular hyphae causes a swollen appearance. Formation of new absorbing roots probably is initiated by the fungus auxin. The branched absorbing roots and the mycelia radiating from the surfaces of mycorrhizae greatly increase the absorbing potential of any particular mycorrhizal root.

The available inorganic ions in the soil have a profound effect on mycorrhizal formation if all other factors are optimal. Within limits, formation of ectomycorrhizae varies inversely with soil fertility. This appears to be especially true when related to the available phosphorus, nitrogen, and possibly potassium in the soil. Addition of inorganic fertilizers to soils may completely suppress formation of ectomycorrhizae.

The hyphae, which frequently radiate some distance into the soil adjacent to the roots, absorb and translocate nutrients from the soil into the root tissues in greater quantities than do roots without mycorrhizae. In exchange, the fungi utilize carbohydrates and other organic metabolites from the root cells. This exchange may be altered by several environmental factors including the availability of nutrients in the soil and light intensity.

When high concentrations of readily available N and P are absorbed from soil and are translocated upward near the source of the photosynthate, soluble carbohydrates are assimilated rapidly during formation of new protoplasm and cell walls in the shoot. The quantity of soluble carbohydrates translocated to and accumulated in the roots and the quantity of the M-factor secreted would be low. Formation of mycorrhizae would not be favored under these conditions. Low light intensity would not favor shoot growth and would result in a similar low amount of soluble carbohydrates in the roots and a similar suppression of mycorrhizal formation.

Current Concepts

Although such a description is obviously very simplified, it illustrates incorporation of several theories on physiological interaction of ectomycorrhizae. It now appears that nearly every hypothesis that has been proposed in an attempt to explain formation of ectomycorrhizae and the metabolic interactions between fungus and host must be qualified and/or expanded. For example, when Hatch (1937) proposed that the internal concentration of nutrient elements in short roots indirectly controlled the infection by mycorrhizal fungi, he was referring to one of several limiting conditions. Others have attempted to attribute the control of mycorrhizal fungi to specific factors, such as concentration of reducing substances in short roots (Björkman 1942) and interactions between fungus auxin and nitrogen (Slankis 1967). I believe we must consider formation of ectomycorrhizae, the physiological influence of

the fungus upon the host, and vice versa, as a complex phenomenon that can be limited or altered by a number of external or internal factors. A dynamic system wherein there is *constant reciprocal availability* of metabolites is necessary to establish and maintain mycorrhizal relationships.

It cannot be denied that mycorrhizal associations are physiologically one of the best examples of balanced reciprocal parasitism in existence. The balanced association continues as long as there is a continuous supply of essential metabolites in the reciprocal exchanges. The exact number and identity of essential metabolites required to maintain the mycorrhizal association is unknown. On the basis of current knowledge, however, certain ones appear to have a decisive influence.

In analytical studies on root exudates, Slankis *et al.* (1964) showed that a wide variety of organic compounds can be secreted by pine roots into the rhizosphere including amino aids, other organic acids and carbohydrates. Bowen (personal communication) reported large differences in the loss of amides and amino acids between *Pinus radiata* seedlings grown under conditions of nitrogen sufficiency, phosphate deficiency, and nitrogen deficiency. He suggested that the exudates could be available for root-infecting fungi, as well as other microorganisms in the rhizosphere. In addition to the above compounds that have been identified in exudates, the metabolite designated as the M-factor (Melin 1954) greatly stimulates mycelial growth of mycorrhizal fungi. The possibility exists that compounds that originate in roots and diffuse into the soil atmosphere could cause stimulation of spore germination and directional growth of hyphae. Regardless, it still appears that the hyphae of ectomycorrhizal fungi in the rhizosphere probably are most dependent on the root for thiamin and carbohydrate.

The fungi that are capable of synthesizing amino acids *in vitro* do not demonstrate deficiencies of this type. Once in contact with the root, a constant supply of a simple carbohydrate, probably glucose, thiamin, and perhaps the M-factor would meet the minimal requirements for maintenance of the fungus. Interruption of the supply would create a deficiency since these compounds would not be readily available in sufficient quantities to satisfy the demands of the fungi. Although a constant supply of available simple carbohydrates in the roots is essential, apparently accumulations of these and other compounds are not essential to maintain the fungus in association with the root. Under conditions comparable to soil, particularly in relation to nitrogen and phosphorus, it is interesting that certain reports (Handley and Sanders 1962; Meyer 1962) do not indicate excessive accumulations of soluble carbohydrates in short roots although carbohydrates are available for utilization by an associated fungus.

Metabolites comprised in part of mineral nutrients are transferred by mycorrhizal fungi into host tissues more efficiently than by root hairs because the hyphae are in contact with a great source of available nutrients (Hatch 1937). Harley (1959) and others have found that accumulation of phosphorus by nonmycorrhizal roots can be as efficient as by the fungus sheath. The absorbing region of the root, however, exhausts the supply of available phosphorus in the vicinity of the root and deficiencies occur in the

plant. Accumulation of phosphorus by the fungus continues as a result of continued mycelial extensions into areas containing available phosphate. The actual release of phosphate to root tissue ordinarily is not rapid, but is continuous over extended periods, even after availability in the soil decreases (Bowen and Theodorou 1967). In addition, the fungus readily transfers ammonium, nitrogen, sulfur, potassium, calcium, sodium, and probably other mineral elements to root tissues. With the possible exception of nitrogen, accumulations of these are not common. Phosphate transport, and perhaps transport of other metabolites through the mantle, is an active metabolic process and requires expenditures of energy. The source of energy no doubt is primarily the simpler sugars or metabolites derived from carbohydrates within the roots. Studies by Bowen and Theodorou (1967), Rovira and Bowen (1968), Bowen and Rovira (1969) and others, have started to elucidate some of the possible biochemical and biophysical transformations in transport of phosphate in ectomycorrhizae.

The sensitivity of mycorrhizal associations to environmental conditions that alter metabolite exchanges can readily be demonstrated by the action of light on the host. Hacskaylo (1965) showed that interruption of photosynthesis of the host in ectotrophs immediately causes a cessation of fruiting of the associated fungus. In other experiments (unpublished), he found that dormancy in pine seedlings caused by an 8-hour photoperiod, although all other conditions are favorable, results in anthocyanin accumulation in the needles, and fruiting of the mycorrhizal fungus ceases. If the seedlings are then placed on a 16-hour photoperiod as soon as growth of the shoot resumes, root growth, mycorrhizal formation, and fruiting of the fungus resumes. Apparently the accumulation of carbohydrates in the shoot on 8-hour photoperiods is caused by interruption of translocation of carbohydrates to the roots and results in subsequent impairment of the mycorrhizal association. These metabolic interactions were somewhat surprising and are being studied more intensively.

Fungus auxin has been proposed as a key metabolite in establishing mycorrhizae (Slankis 1961). The profound effect on morphogenesis and cytogenesis in short roots no doubt is initiated at least in part by fungus auxin. Other than changes in morphology of the short root, Meyer (1962, 1968) suggested that secretion of auxin by mycorrhizal fungi increases the flow of soluble carbohydrates from stem to root. Slankis (1967) showed that unless a continuous supply of auxin is present, short roots elongate and develop root hairs. The reversing action of auxin by tree roots described by Fortin (1967) indicates that unless a continuous auxin supply is maintained by the fungus, the root resumes a regular maturing process. It appears likely that fungus auxin and probably cytokinins (Miller 1967) play a role in preventing maturation and suberization of short roots. These metabolites secreted by the fungus might therefore enhance the ability of fungus pectinase to hydrolyze the middle lamella in the region of the cortex invaded by fungus hyphae. Since auxin and cytokinin actions are linked to protein and RNA synthesis, carbohydrate mobilization, and cell wall extension, their possible roles in mycorrhizal associations should be explored further. It follows, however, that if either auxin and/or cytokinin pro-

duction is interrupted, the root tissues would reject the fungus-host balance in ectomycorrhizae. It appears that available levels of nitrogen in the substrate does indeed cause a break in the "auxin effect" referred to by Slankis (1967). This effect, however, could be indirect, as well as direct, since assimilation of carbohydrates within the host is increased under these conditions.

Conclusions

There is no doubt that ectomycorrhizae are indispensable for completion of the life cycles and survival of the associated organisms comprising the ectotroph. Evolutionary development of mycorrhizal fungi and their hosts has created an interdependency that remains intact as long as external and internal environmental and physiological factors permit an uninterrupted exchange of certain essential metabolites. Interruption or imbalance in the continuous supply of the essential metabolites will impair and eventually destroy the symbiotic association.

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Separate No. FS–296

16.

Studies of Mycorrhizae in Socialist Republics of Europe

S. A. Wilde

As should be expected, the problem of mycorrhizae has received considerable attention in the Soviet Union now engaged in extensive reforestation of war-damaged woodlands and afforestation of prairie soils for amelioration of climatic conditions. A comprehensive account of recent Soviet investigations is given in Imshenetskii and Shemakhanova monographs issued in English in 1967. A very large part of research was concerned with so-called "mycorrhization" of chernozem soils, i.e., artificial introduction of fungal symbionts prior to afforestation by direct seeding. This method was introduced some 70 years ago by G. N. Wissotzky (1902), who was the first to demonstrate on a large scale the importance of mycorrhiza-formers in afforestation of prairie soils. However, this once-solved problem has brought sharp discord among research workers; some of them now claim absence of mycorrhiza-formers in chernozems and insist on artificial inoculation; others deny the need for mycorrhization.

Disagreement of this kind is easily understandable if one takes into account the historic events that took place in the region of Russian and Ukranian prairie-forest. Frequent deforestation by man or fire, and again frequent abandonment of grazing had led to both advance and retreat of forest cover with subsequent degradation and regradation of chernozem soils. As was shown by our studies (Rosendahl and Wilde 1942, Wilde 1954), deforestation, reestablishment of prairie vegetation on once-deforested land, or even prolonged production of farm crops on the same land do not in the least diminish the viability of mycorrhiza-forming fungi.

The problem at hand, therefore, could have been solved by simple clay pot trials that would have saved a great deal of time, expense, and printed paper. However, this must be expected when the interchange of information is curtailed.

On the other hand, Russian microbiologists must be credited for their persistent endeavor to separate root-encased mycelia from extra-matrical rhizospheric organisms, which either directly or through their chelating effects belong among the symbionts of the higher plants (Spyridakis *et al.* 1967).

Studies of mycorrhizae in Czechoslovakia are largely conducted under the leadership of Dr. A. Sobotka of the State Forest Research Institute. Investigations at this center date from the early Fifties and cover both taxonomic and ecological aspects of mycotrophy.

Mycorrhization of soils by broadcast of soil inocula or by pure cultures failed to produce significant results, undoubtedly because inoculations were rendered ineffectual by the presence of autoch-

thonous mycorrhiza-formers which are nearly universally distributed in soils of Bohemia. A different outcome may have resulted from mycorrhization of Moravian chernozems.

Studies of *Alnus glutinosa* and *A. incana* disclosed occasional presence of voluminous, mycoplast-like aggregation of mycorrhizal rootlets. These clusters are formed immediately below soil surface and often under the sporophores of *Lactarius* sp. Root cells of both alders include *Plasmodiophora alni*. The study suggested that alders are endowed with both peripheral and endocellular mycorrhizae.

According to Sobotka, industrial gases find their way below the ground line. Smoke-injured stands of Norway spruce exhibited modified mycorrhizal short roots, an alteration resembling that caused by certain eradicants (Wilde and Persidsky, 1956). Aside from the irregular shape of short roots, industrial air pollution produced thinning, partial reduction, or entire disappearance of the fungal mantle. In the absence of the mantle, hyphae of the hyperthrophied Hartig net extended directly into the soil.

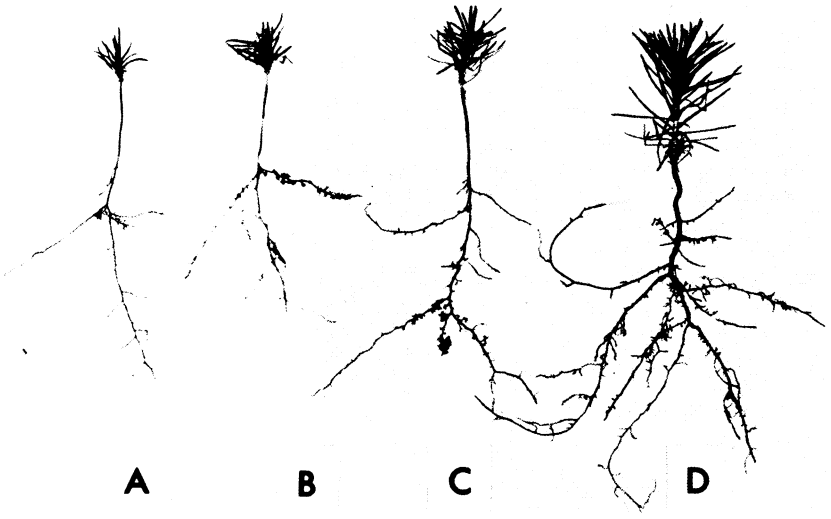
Considerable work was done on the effect of gamma radiation of Co^{60} on mycorrhizal short roots of Scotch pine seedlings (Sobotka 1969). Results, obtained with average duration of 500 to 600 hours a month, are as follows:

	Distance from the source *Meter*	Mycorrhizal short roots *Percent*	Nonmycorrhizal roots *Percent*
Beginning of the second growing season	15	22	78
	24	65	35
	30	65	35
	40	94	6
End of the second growing season	19	15	85
	27.5	16	84
	33	41	59
	39.5	41	59
	47.5	53	47

Šašek and Musílek (1968) observed antiobiotic effect of mycorrhiza-forming basidiomycetes, expressed largely in inhibition of *Rhizoctonia solani*. Simultaneous growth of mycorrhiza formers with seedlings of *Pinus silvestris* failed to increase the antibiotic influence of the lower symbionts. Claims that some fungi (*Russula fragilis*, and *Suillus luteus*) inhibited the development of *Lophodermium pinicolum* are rather puzzling.

Many of the results pertinent to investigation of mycorrhizae in Poland have been available in English through the USDA-NSF translation project. Among other aspects, some of our Polish colleagues have paid a great deal of attention to the morphological characteristics of lower symbionts. This quotation from the report of Wojciechowska (1960), may serve to illustrate the complicated taxonomy of mycorrhiza-forming fungi:

> The spruce (*Picea excelsa* Lk.) forms the following 16 mycorrhizal genera: Aa, Ab, Ac, Ad, Bb, Ca, Cb, Cc, Fa, Fb, Fc, Ff, Ga, Hc, Ia, and Kb.
>
> The genera Aa, Ab, Fa, Fb, Fc, Ff, Ga, and Kb are the most faithful mycorrhizae of the spruce, and their abundance is the highest in the communities studied.
>
> On the average four or five, and occasionally even seven



F-519800

Figure.—Effect of gamma radiation of Co^{60} on the growth of one-year-old seedlings of Scotch pine, *Pinus silvestris* L. at different distances from the source: (A) 15 m, (B) 24 m, (C) 30 m, (D) 40 m. (After A. Sobotka, Forest Res. Inst., Zbraslav-Strnady, Czechoslovakia).

mycorrhizal genera are found on one adult, well-developed spruce.

Morphological effects of gamma radiation are shown in the figure.

I must plead ignorance as the excuse for my inability to make any comment on these findings, except perhaps to paraphrase the commercial slogan: The closer you get, the more complicated it looks.

The German Democratic Republic rendered one, but very significant, contribution to the knowledge of mycorrhiza-forming fungi as well as other soil organisms. It is a 900-page monograph *Boden Biologie* by G. Müller (1961). The scope of information provided by this volume is indicated by more than 2,000 references. It deserves translation into English.

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Separate No. FS–297

17.

Field Inoculations with Mycorrhizal Fungi

J. A. Vozzo

Forest trees of most species are dependent upon mycorrhizae to initiate and support healthy growth. Several situations exist wherein mycorrhizal fungi are not indigenous, and field inoculations with the organisms have been the means of establishing forest trees on many sites that had hitherto been unproductive (Mikola, in press). Conversely, attempts to introduce trees in areas devoid of the symbionts have illustrated the necessity of inoculations.

Growth responses are most evident for ectomycorrhizal hosts in areas devoid of ectomycorrhizal fungi. With endomycorrhiza-forming fungi, which are widely distributed, the purpose of inoculation may be to replace an existing symbiont with one more biologically desirable.

Inoculations with Ectomycorrhizal Fungi

Review

Kessell (1927) failed to establish *Pinus radiata* and *P. pinaster* in Western Australian nursery beds that lacked mycorrhizal fungi. After he added soil from healthy pine stands to the beds, the seedlings grew normally. Oliveros (1932) obtained similar results in the Philippines. In a Rhodesian nursery (Anonymous 1931), pine seedlings grew to about 10 cm and then became chlorotic and stopped growing; addition of fertilizer was unavailing, but inoculation with forest soil containing mycorrhizae produced normal seedlings.

Hatch (1936) was one of the first to inoculate soil with mycelia grown in axenic culture. He planted seedlings of *P. strobus* in 1-gallon jugs containing a 1:1 mixture of prairie soil and sand. After 3 months, the seedlings were small and chlorotic and lacked mycorrhizae. He then inoculated some of the seedlings with mycelia of *Boletus luteus, Boletinus pictus, Lactarius deliciosus, L. indigo,* and *Mycelium radicis nigrostrigosum.* Six months later, the inoculated plants had well-developed mycorrhizae and contained almost twice as much N, P, and K as the uninoculated plants.

In Australia, Young (1936, 1940) demonstrated a remarkable growth response of *P. caribaea, P. patula,* and *P. taeda* when inoculated with *B. granulatus.* Also in Australia, Pryor (1956) inoculated several species of *Eucalyptus* with spores of *Scleroderma flavidum* and obtained 88-percent increases in growth.

The next serious attempts at the artificial syntheses of mycorrhizae were made by Moser, who used pure-culture fungi for field inoculations. He first developed a technique for growing and storing mycorrhizal fungi axenically as pure-culture inocula (Moser 1958a, 1958b). The growth medium was autoclaved peat moss

moistened with a sterilized nutrient solution in an Erlenmeyer flask. He transferred to the flask a single species of mycorrhizal fungus, which grew through the peat moss medium.

Moser (1961) inoculated *P. cembra* and *P. sylvestris* with species of several genera: *Suillus, Amanita, Paxillus, Lactarius, Tricholoma, Leucopaxillus,* and *Phlegmacium*. He used other genera of fungi with *Larix europaea* and *Picea excelsa*. Though he planted the inoculated pines in an area of the Austrian Alps in which many of the fungi were indigenous, he reported differences in stimulation of the growth of pine seedlings inoculated with different species of mycorrhizal fungi. Interpretation of the results was difficult, however, because the uninoculated controls became contaminated by the indigenous fungi.

Goss (1960) made an unsuccessful attempt to inoculate *P. ponderosa* growing in American prairie soil with pure cultures of *Boletus felleus* and *Cenococcum graniforme* isolated from mycorrhizae. He did not attempt axenic technique but instead used open pots in the greenhouse.

Dominik (1961) inoculated sedlings of *P. strobus* in Poland with forest soil that contained *Laccaria laccata, Lachnea* sp., and *Boletus luteus*. Only *B. luteus* formed mycorrhizae. He concluded that forest-soil inoculum is preferable for most practical purposes and that pure-culture inoculation is necessary only when a desirable species of mycorrhizal fungus is lacking in the soil or the existing species is insufficiently virulent.

Laiho (1967) has used *Paxillus involutus, C. graniforme, Corticium bicolor, Boletus* sp., and isolates from ectendomycorrhizal hosts as pure-culture inocula with *P. sylvestris* in Finland under nursery and field conditions. With pure cultures of fungi isolated from ectendotrophs by Mikola (1965), he inoculated four substrates in increasing order of fertility: i.e., soil from peat fields, from clear-cut woodland, from burned-over woodland, and from an arable field. He reported two interesting conclusions. First, the ectendotrophs formed mycorhizal associations more abundantly in the more fertile soils than in soil from the peat fields. This is in conflict with the theory that mycorrhizae offer an advantage to seedlings growing in soils of low N, P, and K availability. Secondly, the seedlings inoculated with the fungus isolated from ectendomycorrhizal seedlings were ectendomycorrhizal in the nursery but readily became ectomycorrhizal when transplanted to the field.

Laiho's results raise the question of host dependence upon ectendomycorrhizal fungi. Are such fungi necessary? If so, can they compete with ectomycorrhizal species under natural conditions? Presumably they have some utility, for Mikola (1965) showed that, under semiaseptic conditions, seedlings inoculated with isolates from ectendotrophs grew better than uninoculated seedlings. As Laiho suggests, an ectendomycorrhizal infection may not promote the establishment of seedlings in woodland soils but may be only a nursery condition.

There have been numerous descriptions of field inoculations by Soviet researchers. Their reports, which are primarily conerned with crop plants and *Quercus* sp. are reviewed in detail by Shemakhanova (1962) and Imshenetskii (1955).

Several areas throughout the world are isolated from the natural

range of specific ectomycorrhizal fungi. Such areas, which include Indonesia, parts of Australia, New Zealand, and Puerto Rico, offer interesting research conditions for field inoculations.

In Australia, ectomycorrhizal fungi were lacking in about 50 percent of the soil samples that Bowen (1963) took in *P. radiata* stands. Subsequently, Bowen (1965) proposed several criteria for inoculating those soils; he emphasized consideration for natural occurrence of mycorrhizal fungi, differences in efficiency of fungal species, competition of introduced fungi with established species, and survival of introduced fungi.

Theodorou (1967) followed these considerations and inoculated *P. radiata* with a pure culture of *Rhizopogon luteolus*. He grew the seedlings in the greenhouse, in nursery soil sterilized with methyl bromide or steam and then inoculated. After 9 months' growth, the inoculated seedlings have more than twice the dry weight of the uninoculated controls (19.33 g and 9.33 g, respectively).

The study was expanded to include pre-culture inoculations of *B. granulatus* and *B. luteus* (Theodorou and Bowen 1967). After 16 months in the field on a loam site, the seedlings inoculated with *B. granulatus* and *R. luteolus* were 46 percent taller than uninoculated controls. *B. luteus* inoculations produced no response.

The primary indication of these Australian inoculations is that introducing fungi that are more efficient than native species may stimulate plant growth.

Prior to artificial mycorrhizal syntheses in Puerto Rico, the island had no pine trees. Synthetic inoculations were initiated by B. J. Huckenpahler of the USDA Forest Service in 1955, when he introduced soil from a pine stand in North Carolina. The mycorrhizal fungi from the soil successfully colonized the seedlings (fig. 1). In all cases, the mycorrhizal pines grew taller than the uninoculated ones and lacked the chlorotic signs typical of uninfected seedlings (fig. 2). Since then, all nurserymen in Puerto Rico have inoculated their seedbeds (Briscoe 1959, Marrero 1962). The pines are then outplanted with well-developed mycorrhizae, and grow up to 10 feet a year.

Huckenpahler showed that field inoculations could be successful, although he used undetermined fungi and impure cultures. The next consideration was a controlled quantitative and qualitative investigation.

Research in Progress

In 1965, Dr. Hacskaylo and I inoculated pine seedlings in Puerto Rico with pure cultures of mycorrhizal fungi, our object being to determine the effect of several species on field-inoculated trees. The host in all cases was slash pine and the fungi were *Cenococcum graniforme, Corticium bicolor, R. roseolus,* and *Suillus cothurnatus.* A mixture of fungi in soil from existing pine stands was also used as a source of inoculum. The growth of inoculated seedlings was compared with the growth of uninoculated seedlings and of uninoculated but fertilized seedlings. As shown in figure 3, inoculated seedlings with mycorrhizae were larger and had a better-developed root system than either the controls or the uninoculated plants that had been fertilized. Applications of commercial fertilizer did not prevent stunting in nonmycorrhizal seedlings.

F-520627

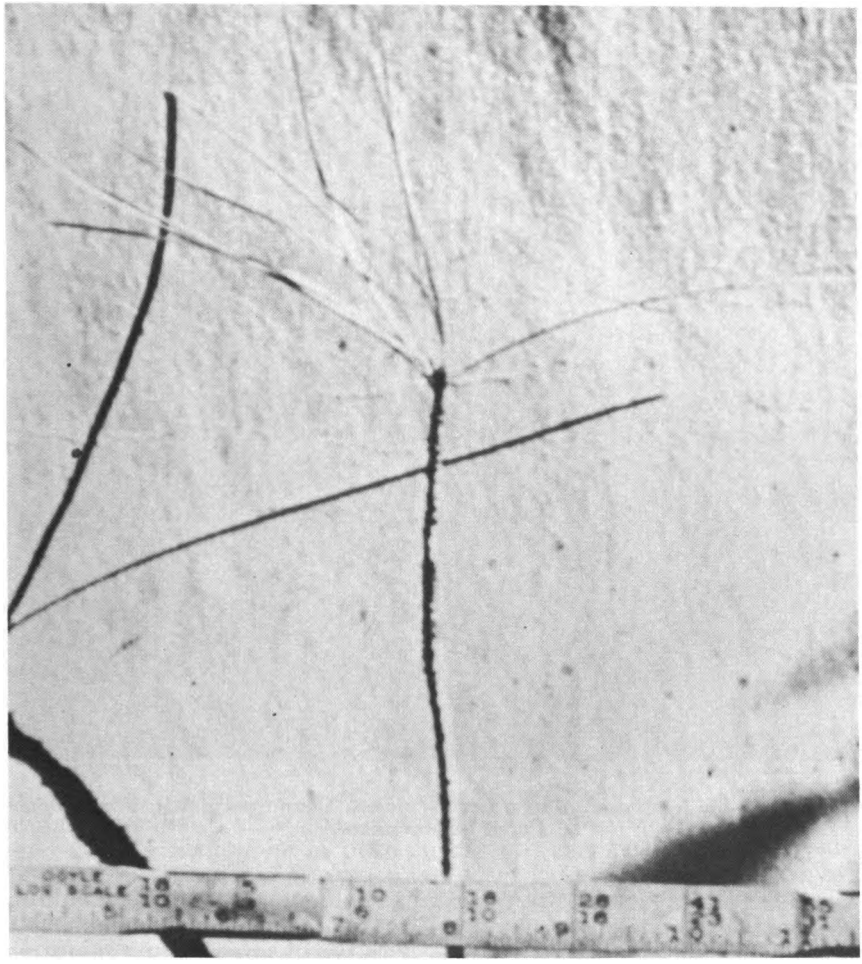
Figure 1.—Five-year-old slash pine seedling inoculated at age 2.

The soil inoculum consistently produced mycorrhizae, while some species in pure culture failed to do so. *Thelephora terrestris* was introduced with the soil inocula and readily formed sporophores and mycorrhizae in the nursery. *Corticium bicolor* formed mycorrhizae more readily than the other four fungi, while *Cenococcum graniforme* did not become a mycorrhizal associate in the field or nursery. As pure-culture inocula all five fungi formed mycorrhizae under axenic culture in the laboratory.

Complete results of the Puerto Rico inoculations will be published later.

Introducing controlled inocula into areas with no indigenous mycorrhizal fungi may involve many complications. Quarantine regulations and the difficulty of maintaining viability during transport often restrict the use of soil inoculum. Customs officials and agricultural inspectors are usually considerate of research needs if prior arrangements are officially made, but they cannot allow indiscriminate passage of raw soil and plants. A prime objective of the Puerto Rico inoculations was to determine an acceptable method of transporting pure cultures viably and legally.

Modifying the technique described by Moser (1958a), we trans-



F-520628

Figure 2.—Uninoculated slash pine seedling 5 years old.

ferred pure cultures of mycorrhizal fungi to a peat moss-vermiculite medium wetted with a nutrient solution. The medium was in polypropylene cups with paper tops. *Rhizopogon roseolus, C. graniforme,* and *S. cothurnatus* were aseptically macerated and pipetted into the medium. They grew well and in 4 to 6 weeks spread through the medium in each cup. *Corticium bicolor* treated in the same manner survived but would not colonize the medium. After several repetitions failed, *C. bicolor* was successfully cultured from whole pieces of mycelia rather than from macerations.

The cultures in the cups were stored for up to 5 months in a cold room (2° C); the cups were taken from storage as needed, packed into cardboard cartons, and shipped by airfreight to Puerto Rico. This medium has supported the same species in culture for 1 year in glass flasks at room temperature.

Besides allowing considerable handling, cups offer several other

Figure 3.—Slash pine seedlings. I=inoculated, U=uninoculated, F=uninoculated but fertilized.

advantages: (1) Since they contain small quantities of inoculum, they may economically be opened for one or two inoculations, (2) if a cup becomes contaminated, only a small amount of the entire inoculum source is lost, and (3) the cups are durable shipping units.

A modification of this technique was made when we needed a small amount of pure-culture inocula for use immediately after shipment. Large pieces of mycelia were introduced into the same medium but in glass flasks. After mycelia had spread through the substrate, the contents of several flasks were aseptically transferred into sterilized plastic bags for transport. The bags are more liable to contamination and desiccation than cups and are much more fragile to ship. However, they are satisfactory when small amounts of inoculum are to be used before contaminants become well established.

Inoculations with Endomycorrhizal Fungi

Review

The phycomycetes responsible for vesicular-arbuscular endomycorrhizae are commonly found in most soils throughout the world (Gerdemann 1968). Their distribution in soils is much more cosmo-

politan than that of ectomycorrhizal fungi. Field inoculations, therefore, would be more likely to involve species substitution or fortification through increasing population levels of existing fungal symbionts.

Peyronel (1923) first suggested that the vesicular-arbuscular fungi were *Endogone* species. But proof was lacking until Mosse (1956) found *Endogone* sporocarps attached to mycorrhizal strawberry roots. With the sporocarps, she inoculated uninfected strawberry roots and synthesized endomycorrhizae in pot culture. Researchers had previously used soil inoculum to infect roots, but the endophyte had not been identified.

Gerdemann (1955) devised a wet-sieving and decanting technique to obtain several *Endogone* species from soil. This procedure recovers sufficient spores to provide inoculum for mycorrhizal syntheses. Gerdemann has inoculated plants in the greenhouse and synthesized mycorrhizae on corn, onions, strawberry, red clover, and yellow-poplar.

Baylis (1967), in trials with *Griselinia* in New Zealand, grew seedlings in soil known to have endomycorrhizal fungi. He later transferred the seedlings to sterilized soil, where mycorrhizal seedlings grew better than seedlings that lacked such associations.

Neither of these techniques involve pure-culture inocula. Barrett (1961) reported isolating *Endogone* (*Rhizophagus*) from mycorrhizae and successfully reinoculating to synthesize vesicular-arbuscular mycorrhizae. Others, however, have been unable to establish infections from his isolates. The difficulties of establishing pure cultures of obligate organisms on synthetic media are not peculiar to the study of mycorrhizal inoculations.

Following her work on strawberries, Mosse (1962) used aseptic procedures to inoculate clover seedlings grown on agar with surface-sterilized *Endogone* spores. This was the first confirmed report of an *Endogone* in pure culture on aseptically grown seedlings. Still, the infection was unsuccessful unless accompanied by culture filtrates, EDTA, pectinol, or a *Pseudomonas* sp. Mosse proposed that the bacterial species produced proteolytic enzymes that promoted infection. However, soluble nitrogen added to the medium completely inhibited root penetration.

To test the possibility of organisms adhering to *Endogone* spores used as inoculum, Clark (1969) used live and dead spores as inocula on sugar maple, boxelder, yellow-poplar, and white ash. Spores killed by piercing with a needle were added to the sterilized soil in control pots in the same number as living spores were added to inoculated pots. This assured a similar microflora in both the inoculated and control pots. Seedlings in pots with the pierced spores never exhibited the growth response observed in plants inoculated with live spores.

An outstanding review of inoculation experiments with endomycorrhizal fungi was recently published by Gerdemann (1969). He describes many investigations made in sterile or nonsterile soil, and with *Endogone* spores or mycorrhizal roots as inocula. The review is comprehensive, and it would be repetitious to dwell on it.

In general, literature on endomycorrhizae supports several conclusions. In nearly all instances of sucessful inoculation, there was a reported growth increase of mycorrhizal over nonmycorrhizal

plants. Increases were characterized by shoot elongation, prolific root generation, and lessening of chlorosis or similar morphologic symptoms. In some instances, infected plants made no obvious growth response but nevertheless developed more dry weight than controls (Daft and Nicolson 1966). Other physiological data include an increase in phosphorus uptake from soil of mycorrhizal maize over uninfected maize (Gerdemann 1965). Gray and Gerdemann (1967, 1969) report that mycorrhizal sweetgum, yellow-poplar, and onion accumulate more P^{32} from soil in pot culture than do nonmycorrhizal plants.

The ability of vesicular-arbuscular mycorrhizae to enhance phosphorus uptake may well be their prime value as inoculants for plants in the field. At this time, there is no evidence to suggest that endomycorrhizae function differently from ectomycorrhizae in increasing nutrient uptake.

Research in Progress

Recently, Dr. Gerdemann and I have examined *Endogone* in soil samples from *Populus deltoides* plantations in the Mississippi Delta. Planting sites included old pasture, cleared woodland, and cottonfield on high ground and batture land.[1] The soil is predominantly of the Sharkey series, which is high in montmorillonite, is nonacid in reaction, and contains about 2 to 3 percent organic matter. Texture is typically clay, and physical condition is firm to plastic with varying amounts of moisture. Using Gerdemann's wet-sieving and decanting method, we first isolated only small numbers of *Endogone* spores. Our early impressions were that perhaps these forest soils were sparsely infected with *Endogone*. However, a small-spored species (up to about 120 μ in diameter) would occasionally appear. Working with a small-meshed sieve, we realized that *Endogones* were indeed in these soils, but we found only species with relative small spores. Further observations suggested that two species were present, *Endogone macrocarpa* var. *geospora* (as described by Nicolson and Gerdemann (1968) but with much smaller spores), and *.E. fasciculata.*

Since those observations were made, we have designed field inoculations to test effects of several *Endogone* species qualitatively. We do not expect to find striking differences between inoculated and uninoculated trees. There is no reason to believe that natural infection has not reached the host species; however, the *Endogone* distribution is often sparse. Johnson grass (*Sorghum halepense*), a common weed, is also an endotrophic host of *Endogone* and has infiltrated most plantations. Thus, *Endogone* from forest trees or Johnson grass is potentially available as natural inoculum. These investigations involve substituting *Endogone* species by artificial soil inoculation. By introducing additional species to the host, we may be able to increase efficiency of nutrient uptake (Gerdemann, 1969).

Discussion and Conclusions

Several questions brought out here require further testing by field inoculation. Seedlings inoculated with pure cultures do not

[1] Batture land is the strip between the Mississippi River and its levees and hence is subject to periodic flooding.

respond as well as those receiving a soil inoculum. This was shown by Mikola's and Laiho's work with a pure-culture fungus isolated from ectendomycorrhizae in Finland and our own inoculations in Puerto Rico. The soil inoculum may contain a variety of fungus species that form mycorrhizae on the host plant, but there seems to be more of a synergistic response from the natural mixture than a direct effect by any one species in it. Several fungi, both mycorrhizal and nonmycorrhizal, have been isolated from mycorrhizae formed by soil inoculation. The isolated mycorrhizal fungi are not as vigorous or capable of stimulating the host as when they were a part of the soil mixture. We do not know the influence of all nonmycorrhizal fungi in the natural rhizosphere.

Under field conditions, none of the pure-culture inoculations formed the quantities of mycorrhizae that the soil mixture produced. Perhaps the mycorrhizal relationship comprises more than the symbiosis of two organisms. As mentioned earlier, Mosse (1962) failed with pure-culture inoculations of an endomycorrhizal host but succeeded when she introduced a third organism.

From a practical viewpoint, field inoculations with mycorrhizal fungi have proven their value under specific conditions. As we expand our understanding of symbiotic ecology, they will become more valuable to researchers and planters.

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18.

Critique

A. B. Hatch

I wish to compliment Dr. Hacskaylo and Professor Gerdemann on the success of this First North American Conference on Mycorrhizae.[1] I feared that most of the contributions here would consist of reviews and revisions of previously published research and theories. Instead, a wealth of original data has been presented and excellent new techniques have been described. A year ago, when I chaired a mycorrhizal symposium at the ANZAAS Congress in New Zealand, I felt quite strongly that European and ANZAAS countries were leading the world in mycorrhizal research. Today, as a result of this Conference, I know that in North America we are also in the mainstream of progress.

Unfortunately, these proceedings cannot contain all of the most important features of this conference. It cannot include Dr. Zak's magnificent color photographs of Douglas-Fir mycorrhizae which demonstrate far better than all my photographs, drawings, and words combined (Hatch, 1937), the enormous increase in absorbing surface area which is induced in lateral roots by ectomycorrhizal fungi. His illustrations clearly depicted his suggested scheme for classifying Douglas-Fir mycorrhizae.

These proceedings also will not include Dr. Hacskaylo's illustrations of his study of *Thelephora terrestris* (Ehrh.) which demonstrated with precision and conclusiveness that this mycorrhizal fungus, at least, is wholly dependent upon the tree host for the energy sources which enable it to produce sporophores.

Dr. Marx's illustrations of the "mycorrhizal fungus spore-free room" being built in his greenhouse will not appear in the conference publication. Such a room will eliminate the requirement for pure culture techniques in many experiments. A somewhat similar, but primitive exclosure enabled me to first demonstrate that nutrient absorption and tree growth are made possible by adding pure cultures of ectomycorrhizal fungi to soils lacking those fungi (Hatch 1936).

At first, I was surprised to find that so much time was to be devoted to the taxonomy of the ectomycorrhizal fungi. However, I understood why on hearing the presentations of Professor Smith and Doctors Trappe and Zak. They pointed out that the known physiology of ectomycorrhizal fungi varies so widely that it is inconceivable that their effects on tree nutrition in different soils and different climates do not also vary. It is obvious, therefore, that exact identification of the fungi used in experimental work and synthesis experiments is essential. Further, means for identifying the mycorrhizal fungi in the absence of sporophores, as de-

[1] This critique was given at the close of the conference. It is based on the invitational papers only.

scribed by Doctors Zak and Trappe, can be of the greatest importance in field studies. The magnitude of this undertaking, however, seems so great when one considers the numbers of fungi and hosts which produce ectomycorrhizae that I suspect a century may pass before a significant number of fungal associates in mycorrhizae can be identified by mycelial characteristics alone.

I was especially interested in Dr. Trappe's account of the association of ascomycetes with ectomycorrhizae. He proposed that several species of *Elaphomyces* may produce the jet black mycorrhizae I first synthesized in pure culture between *Pinus strobus* L. and *Cenococcum graniforme* (*Mycelium radicis nigrostrigosum*, Hatch 1934). He pointed out that the peculiar mosaic of stellate hyphal clusters characteristic of these ectomycorrhizae, and apparently of no others, is replicated in *Cenococcum* sclerotia and in the peridial layer of *E. anthracinus* and other *Elaphomyces* species. According to Dr. Trappe, *C. graniforme* has typical ascomycete septal pores rather than the complex dolipores of the basidiomycetes. If species of *Elaphomyces* are the perfect stage of *C. graniforme*, the fact should be readily confirmed since *C. graniforme* is one of the easiest ectomycorrhizal fungi to isolate from mycorrhizae. Therefore, it should be readily cultured from the sporocarps of *E. anthracinus*.

In his discussion on the taxonomy of endomycorrhiza-producing fungi, Professor Gerdemann said that endomycorrhizae of the vesicular-arbuscular type (VA mycorrhizae) are by far the most common. Much less is known about them than ectomycorrhizae and septate endomycorrhizae because until recently neither the identity of the fungi producing them nor the method of culturing the fungi was known. Today, however, species of *Endogone* are known to be, at least, the primary endophytes. Professor Gerdemann has provided a key to all seven named and three unnamed species of *Endogone* known to produce VA mycorrhizae, and has given descriptions and references for each species. The wet-sieving and decanting technique developed by Dr. Gerdemann for assaying soils for *Endogone* spores and his technique for culturing the spores with maize plants in pot cultures have enabled him to establish life cycles of the fungus, its host ranges, and to determine the effects of the fungus on nutrient absorption.

Both Professor Gerdemann and Dr. Gray spoke on the physiology of VA endomycorrhizae. They, Professor G. T. S. Baylis (1962, 1967), and several other workers have found a relationship between soil fertility and the development of VA mycorrhizae which seems to parallel the situation in ectomycorrhizae. Phosphorus uptake especially appears to be greatly increased by VA mycorrhizal infection as it is in ectomycorrhizae. Professor Baylis (1962, p. 199) concluded that "... the same fundamental mechanism may underlie growth stimulation by both types of mycorrhiza." However, Professor Gerdemann cautioned us not to accept this conclusion without resevations. He and Dr. Gray found that maize will not develop normally in soils which will produce 250 bu. of corn per acre in the absence of infection by *Endogone*. It is interesting that maize plants in fertile soil, by contrast to ectomycorrhizal tree-seedlings, do not grow rapidly and absorb large quantities of soil nutrients. If the same mechanisms are at work in both ectomycor-

rhizal fungi and VA endomycorrhizal fungi the explanation well might be a mild imbalance in nutrient availability (Hatch 1937).

In one of the most interesting papers presented, Dr. Marx summarized his studies on the deterrent effects of ectomycorrhizae on root pathogens. He proved that mycorrhizal fungi inhibit the invasion of *Phytophthora cinnamomi* in feeder roots. One mycorrhizal fungus that Dr. Marx studied, *Leucopaxillus cerealis* var. *piceina* (Peck) ined., produced a very potent antifungal and antibacterial chemical, diatretyne nitrile. While this antibiotic inhibited the growth of 92 percent of the pathogens tested in culture and the mycorrhizae produced by this fungus were completely free of invasion by *P. cinnamomi*, it could not be determined whether this was due to the chemical or to the mechanical effects of the mantle or Hartig-net or both. These effects may be difficult to separate in future experiments.

Professor Davey suggested that the absence of additional organisms in monoxenic synthesis experiments may prevent recalcitrant fungi from producing mycorrhizae. This is known to be the case in one species of *Endogone* (Mosse 1962). Professor Davey suggested that more attention be given to the use of dixenic and even polyxenic cultures. If this is found to be necessary, I believe that the easiest solution would be use of a "mycorrhizal fungus spore-free room" of the Marx type together with, (1) natural soils which lack ectomycorrhizal fungi but contain a wide range of other organisms, (2) partially sterilized soils, or (3) other types of substrates containing the desired organisms.

In Dr. Riffle's paper on mycophagous nematodes, he reported on a species of *Aphelenchoides* isolated from *Pinus ponderosa* Laws. He found that nematodes fed and reproduced on 50 of 53 known or suspected mycorrhizal fungi. Therefore, it might be advisable to look for fungus-eating nematodes if mycorrhizae do not develop in spore-free rooms.

Professor Wilcox expanded our knowledge of ectendomycorrhizae. He confirmed Mikola's (1965) observation that one-to-two-year-old nursery seedlings are ectendomycorrhizal exclusively, and that older seedlings in the nursery and in plantations become ectomycorrhizal. Professor Wilcox's photographs and photomicrographs of *Pinus resinosa* Ait. root systems were especially good. His descriptions of ectendo- and ectomycorrhizal infection in long roots were interesting, yet one point, I believe, might be re-examined. Professor Wilcox apparently believes that mycorrhizal infection of long roots, especially the small-diameter, subordinate roots, tends to inhibit elongation. It seems to me that these were probably "feeble roots" (Aldrich-Blake 1930, p. 24) from the beginning and that the total elongation, due to development of two or more tips, may be increased rather than inhibited by infection (Hatch and Doak 1933).

Professor Bilan presented a very useful summary of the inherent characteristics of a wide variety of tree roots. He also discussed the influence of the habitat environment, especially soil characteristics, moisture, and temperature, upon these inherent characteristics. Provenance was also reviewed as it affects intra-species variations in root systems. Apparently, we should be as careful in de-

fining the source of tree seed used in mycorrhizal studies as we are in identifying fungal associates.

In Professor Voigt's review, the perennial question of nitrogen fixation by mycorrhizal fungi was discussed, and he stated, "There is no good evidence that mycorrhizal fungi are themselves directly involved in nitrogen fixation, but there is some indication that the mycorrhizal system does somehow stimulate fixation." By mycorrhizal system, Professor Voigt apparently meant, "many coniferous forest systems." He concludes that in such forests the sum of the known nitrogen fixing rhizosphere microorganisms and symbioses, " . . . seems inadequate to explain the rates of nitrogen accumulation . . . ." I would hazard the guess that it would be more profitable to search for other nitrogen-fixing systems in such forests than to continue experimentation using the most sensitive isotope methods in aseptic cultures.

A few years ago, after efforts to establish pines in Puerto Rico had failed, a splendid opportunity existed for inoculating seedlings with pure cultures of these fungi. In 1964, Dr. Vozzo, several others in this conferene room, and I, took part in an International Union of Forest Research Organizations' Mycorrhiza Working Group field examination of the early experiments on this island. In 4 years, *Pinus caribaea* var. *hondurensis* had grown 20 feet high after being inoculated with forest soils from the United States. Thus, I expected that Dr. Vozzo would report that pines, inoculated with pure cultures of mycorrhizal fungi were now also 20 feet high compared to uninoculated controls of two or three inches. Unfortunately, the early experiments were failures. The studies that Dr. Vozzo described were started after our visit to the island in 1964 and time has been insufficient to produce such spectacular differences in tree growth. Dr. Vozzo states, ". . . inoculated seedlings with mycorrhizae were larger and had better developed root systems than either the controls or the uninoculated plants that had been fertilized. Applications of commercial fertilizer did not prevent stunting in nonmycorrhizal seedlings."

The failure of plants to respond to fertilization in natural soils in the absence of mycorrhizae remains a mystery in both ecto- and endotrophs. Tomatoes and corn and tree-seedlings can be grown in hydroponic systems without the appearance of nutritional deficiencies or stunting, so it would seem unlikely that mycorrhizal fungi perform some unique internal physiological role in, for example, the metabolism of phosphorus, as suggested by several students of mycotrophy. It follows that the failure of mycotrophic plants to grow normally in highly fertile soils in the absene of mycorrhizal infection remains today one of the most fundamental problems for research in this field.

Unfortunately some of us, and certainly this writer, were unfamiliar with many of the mycorrhiza investigators in the Socialist Republics of Europe mentioned by Professor Wilde. My only comment on his presentation is to question his statement that, " . . . deforestation, re-establishment of prairie vegetation on once-deforested land, or even prolonged production of farm crops on the same land, do not in the least diminish the viability of (ecto) mycorrhizal fungi." The habitats of these soils are perhaps always showered with spores of the Hymenomycetes which inhabit nearby

forests (Melin 1917). The question of the longevity of most or all ectomycorrhizal fungi in soils in the absence of their vascular associates, remains unanswered.

One of the highlights of our conference was Professor Miller's paper on the production of cytokinins by ectomycorrhizal and pathogenic fungi. Dr. Miller's interest in mycorrhizal fungi stemmed from the fact that both ectomycorrhizal fungi and cytokinins cause enlargement of root cortical cells and the formation of nodules on tobacco roots. He found that four mycorrhizal fungi and an unidentified ectendomycorrhizal fungus produced cytokinin, but that *Cenoccum graniforme* (according to Trappe, an ascomycete), *Thelephora terrestris*, and 22 nonectomycorrhizal fungi did not. The subject is too new and interpretation too uncertain for me to comment on the possible roles of these chemicals in mycotrophy and pathogenesis.

To anyone who has tested the ability of fungi to produce mycorrhizae in pure culture, the report by Dr. Palmer brings together a wealth of useful (and to me, nostalgic) techniques. My techniques, back in 1930, were learned directly from the great maestro, Professor Elias Melin. According to Dr. Palmer's paper, today's techniques are basically unchanged, but greatly refined and better defined. Antibiotics, which I have been exploring and producing for the last twenty-odd years, are now used to reduce bacterial contamination in sporophore tissue isolations and in the recovery of the ectophyte from mycorrhizae. Many more vitamins and growth-stimulating extracts from roots are sometimes employed to bring recalcitrant species into culture, but malt extract is still an almost essential ingredient of culture media. In my experience, back in the 30's, Liebig's Malt Extract, condensed at low temperatures under vacuum and imported from Apoteksvarucentral Vitrum, Stockholm, Sweden, was far superior to American brands. The problem of what is missing in all artificial media to bring many mycorrhizal fungi into pure culture is still another mystery and one of the most challenging research undertakings in both the ecto- and endomycorrhizal fields.

I am somewhat reluctant to comment on Dr. Hacskaylo's review of "Metabolite Exchanges in Ectomycorrhizae." This is partly because whatever I say will add little to the acclaim he deserves, with Professor Gerdemann, for the superb organization of this Conference. However, it is my feeling that Dr. Hacskaylo has synthesized such a broad and all-inclusive hypothesis to explain the ectomycorrhizal habit, tying it to so many, if not quite all, theorists in our field, that there is almost no one thing that one can attribute to Dr. Hacskaylo exclusively. Now I think this inability to criticize Dr. Hacskaylo's theory will certainly frustrate future students of mycotrophy. Few tasks have afforded students of mycorrhizae more armchair recreation than taking old theories apart and conjuring up new ones. I think Dr. Hacskaylo will be immune from these attacks!

Dr. Slankis reported a series of experiments designed to explore Björkman's theory that a surplus of soluble sugars does not occur in seedling roots grown in high concentrations of nitrogen and phosphorus. Dr. Slankis reported finding an abundance of sugar in roots regardless of nutrient levels. He then told of his well-known

work on auxins. He believes that the specific physiological conditions which induce ectomycorrhiza formation are a result of the auxins produced by the higher plant. If this is correct, it would seem that high, balanced levels of nitrogen, phosphorus, and other internal seedling nutrients must inhibit the production of auxins (Hatch 1937, p. 93).

Among the research reports presented at this Conference there was a conspicuous absence of work on the energy relationships between fungi and host. Considerable improvement in our understanding of the carbohydrate physiology of ectomycotrophy has been made during the last decade, and some mention of this work should have been made at this Conference.

Professor Harley (1968) has provided a summary of carbohydrate studies in the second edition of his book, *The Biology of Mycorrhiza,* which is now in press. The studies were carried out by Harley & Jennings, 1958; D. H. Lewis, 1963; and Lewis and Harley, 1965. They have shown that there are several classes of reducing substances in ectomycorrhizae of beech, and only about 50 percent of these are revealed by the chemical analyses used by previous workers (Björkman 1942, 1944). Host tissue contains sucrose, glucose, and fructose, all of which are readily utilized by the fungus. Alternatively, mannitol, trehalose, and glocogen (the principal one and insoluble) are important storage carbohydrates of the fungal mantle and these cannot be used by the host.

Lack of reciprocal flow of stored carbohydrates from fungal tissue to the host consitutes a " . . . sink into which carbohydrates are passed and from which they may be taken and utilized by the fungus, but not by the host" (Harley 1968, p. 96). It follows that chemical analyses of reducing substances in mycorrhizae of the kinds which have been used in the past are suspect and so are conclusions based upon these analyses.

Many other advances in knowledge of mycorrhizae have been made during the 35 years since I last worked in this field. Not the least among these has been the reassessment of the confused picture left by Dr. Rayner (1927) of mycotrophy in the Ericales. In the second edition of his book, Professor Harley (1968, p. 174) marshalled many factors indicating that Ericoid mycorrhizae are very similar to ectomycorrhizae of forest trees. He summarized them: "These results are similar in many ways to those obtained with forest trees, and show that the intensity of infection decreases in soils which are well supplied with nutrients and that it depends in some measure upon the internal nutrient status of the host."

Studies on excised beech ectomycorrhizae by Professor Harley's group at Oxford have also added to knowledge of mycotrophy in recent years. This work is summarized in the second edition of his book and will not require further comment here.

Finally, I wish to pay tribute to the relatively recent contributions of Professor Elias Melin. Using radioisotopes, the most powerful and elegant of modern tools, Professors Melin and Nilsson (1950, 1952, 1953a & b, 1955, 1957) confirmed the transfer of phosphorus, potassium, nitrogen (both organic and inorganic), and calcium from isolated substrates through fungal mycelia and mycorrhizae to tree seedlings which had no other contact with the substrates containing the radioactive materials. They also dem-

onstrated the transfer of radioactive carbohydrates synthesized by the seedlings from C^{14} in the leaves to the mycorrhizal fungus. Melin et al. (1958) also demonstrated translocation of cations to seedlings of *Pinus virginiana* through mycorrhizal mycelia.

In ending this "Critique", may I add that these are merely among the latest of Professor Melin's researches. Over 50 years ago, in 1917, he published his first, a Doctoral Thesis, and only a few years later, he was recognized as the world's leading authority on ectomycorrhizae.

It has been a great pleasure for me to take part in this Conference and to join you in dedicating our proceedings to my former professor and friend, Elias Melin, the pioneer and modern master of experimental knowledge of ectomycorrhizae.

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19.

Contributed Papers

Separate No. FS-299

Forest Mycology and Forest Communities in South America. II. Mycorrhiza Sociology and Fungus Succession in the *Nothofagus dombeyi-Austrocedrus chilensis* Woods of Patagonia

Rolf Singer

In studies on mycorrhizal ecology, it is essential to identify the symbiotic components in each geographic region. Our list of obligatory mycorrhizal fungi and cormophytes, which form ectomycorrhizae and ectendomycorrhizae (Singer and Morello 1960) is still valid, but our most recent observations show that the genus *Eucalyptus,* Myrtaceae and the genus *Shorea,* Dipterocarpaceae should be added to that listing.

My method of determining whether a fungus is an ectotroph component (as outlined in 1951 and described in 1964) starts with a taxonomic generalization supported by isolated laboratory and field observations. The phytogeography and mycosociology of the assembled taxa are examined individually. First, the geographic area of the fungus is determined to see if it falls wholly inside the combined area of all obligatory ectotrophs. If the species appears regularly in the ectotroph-dominated woods, I determine if it disappears in the adjacent anectotrophic woods or in the woods from which the ectotroph tree has been removed. These methods are applicable to all elements of the flora in a region and express the actual role of these organisms in nature much more accurately than does synthesis alone. Synthesis is used rather as a last, confirming step to exclude the possibility of error in other procedures.

Facultative ectotroph-formers, i.e., fungi which form ectomycorrhizae only occasionally, do not appear in our lists of specialized species, although some, like *Paxillus involutus,* have enormous significance in the succession and renovation of forest communities. Because these fungi are capable of fruiting in the absence of an ectotroph tree, they may inhabit an ectotroph-dominated wooded area and an adjacent anectotrophic woods as well. This behavior may be attributed to a capacity to utilize polysaccharides for nutrition (Norkrans 1950) and, perhaps, to other factors now not fully understood.

The temperate and frigid regions of southern South America have numerous adjacent ectotrophic and anectotrophic woods. This

[1] This research was supported by grants from the National Science Foundation, Washington, D.C., Consejo Nacional de Investigaciones Cientificas y Técnicas, Buenos Aires, and the Facultad de Ciensias Exactas, Universidad de Buenos Aires, Argentina.

situation is typical in certain large national parks (particularly P. N. Nahuel Huapi, Río Negro and Neuquén, Argentina) where forests remain reasonably undisturbed and where forest fires are mostly of natural origin. In these areas, the exact time of a fire as well as the type of vegetation destroyed by it are completely and accurately recorded.

Mycoecological studies were initiated in certain of these areas which were covered by a *Nothofagus dombeyi-Austrocedrus chilensis* climax forest, and in adjacent burned areas where repopulation stages ranged from 12 months, the *Funaria* stage (cf Moser 1949), to 20 years. We obtained data by the "Moser Method" (Moser 1959), working with fully defined associations (Oberdorfer 1960; Dimitri 1964, Eskuche ined; Sarmiento ined). All fructifications of Agaricales and soil and litter inhabiting macromycetes within the defined areas were collected, identified, sorted out, and counted according to substratum.

In a humid western subzone covered by a *N. dombeyi-A. chilensis* forest climax, the following fungal species by percentage distribution were observed during all seasons, 1950–1966:

Humicolous and Litter Species:
43 percent

Hygrocybe singeri
Laccaria tetraspora
Lyophyllum langei
Lyophyllum fumosum
Clitocybe angustissima
Clitocybe nothofageti
Clitocybe obscuratipes
Lepista diemii
Ripartites tricholoma
Gerronema fibula
Gerronema minutum
Melanoleuca luteolosperma
Melanoleuca melanosarx
Campanella dendrophora
Collybia subhybrida
Collybia sp. aff. *butyracea*
Marasmiellus couleu
Macrocystidia cucumis
Mycena albogrisea
Mycena amygdalina
Mycena austroavenacea
Mycena haematopoda
Mycena microleuca
Mycena phyllogena
Mycena pura
Mycena sp.
Fayodia striatula
Xeromphalina austroandina
Hydropus lipocystis
Leucoagaricus erythrellus
Leucoagaricus atrofibrillosus
Melanophyllum echinatum
Lepiota cristata
Lepiota neuquenensis
Cystolepiota australis
Cystolepiota marthae
Cystolepiota rosea
Coprinus atramentarius
Coprinus disseminatus
Coprinus subimpatiens
Psathyrella dactylocystis
Psathyrella patagonica
Psathyrella polycystidiata
Psathyrella subandina
Pholiotina verrucispora
Conocybe cryptocystis
Descolea recedens
Psilocybe araucana
Psilocybe fuegiana
Psilocybe montana
Pholiota megalosperma
Pholiota microcarpa
Pholiota psathyrelloides
Pholiota subflammans
Galerina inconspicua
Galerina patagonica
Galerina victoriae
Tubaria furfuracea
Clitopilus hobsonii
Rhodocybe caelata
Rhodocybe himantiigena
Rhodophyllus chloropolius
Rhodophyllus fuscifolius
Rhodophyllus lazulinus
Hydrophoropsis aurantiaca
Lentinellus omphalomorphus

Clavulinopsis hexaspora
Clavulina cristata
Clavariella patagonica
Clavariella stricta
Hysterangium separabile
Thaxterogaster magellanicum
Weraroa spadicea
Simocybe curvipes
Simocybe olivaceiceps
Mycenastrum corium
Lycoperdon sp.
Gyromitra antarctica
Morchella conica
Morchella sp.
Underwoodia fuegiana
Sowerbyella thaxteri
Jafnea tasmanica var. *singeri*

Lignicolous Agarics: 16.5 percent

Polyporus gayanus
Polyporus maculatissimus
Polyporus melanopus
Clitocybe pleurotus
Clitocybe subleptoloma
Armillariella montagnei
Armillariella novaezelandiae
Armillariella sparrei
Clitocybula mellea
Campanella dendrophora
Panellus longinquus
Mycena galericulata
Mycena haematopoda
Mycena pseudoalnicola
Mycena pseudovulgaris
Xeromphalina austroandina
Pluteus flammipes
Pluteus jaffuelii
Pluteus spegazzinianus
Psathyrella polycystidiata
Melanotus gayi
Melanotus patagonicus
Naematoloma sublateritium
Pholiota baeosperma
Simocybe pantelaeodes
Galerina patagonica
Tubaria furfuracea
Crepidotus applanatus
Crepidotus brunswickianus
Crepidotus nephrodes
Crepidotus sphaerosporus
Crepidotus sp.

Obligatory Ectotroph-Formers: 38 percent

Tricholoma cortinatellum
Tricholoma diemii
Tricholoma patagonicum
Porpoloma sejunctum
Porpoloma terreum
Amanita diemii
Amanita umbrinella
Inocybe erythrobasis
Inocybe bridgesiana
Inocybe diemii
Inocybe (15 sp.)
Hebeloma moseri
Cortinarius (37 sp.)
Russula fuegiana
Russula major
Russula nothofaginea
Paxillus defibulatus
Paxillus boletinoides
Ramaria holorubella
Ramaria inedulis
Ramaria strasseri

Nothofagus Parasites: 1 percent

Bondarzewia guaitecasensis
Hybogaster giganteus

Dung Inhabiting Agarics: 1.5 percent

Panaeolus campanulatus
Panaeolus sphinctrinus
Stropharia semiglobata

In a burned area in the same subzone, a new fungal population was evident (see table 1). Other significant observations are as follows:

(1) After the forest fire, the percentage of fruiting bodies of obligatorily specialized, ectotroph-forming fungi goes down to the level of the anectotrophic forests (0%), and remains at this level until the first seedlings of *Nothofagus* have become established and have grown up to form a dense brush.

(2) During the formation of the first seedlings of *Nothofagus* from windblown seed and during the following several years, the symbiont of *Nothofagus* in the area is a facultative ectotroph-

former, *Laccaria tetraspora*. In other regions of Patagonia and South Chile, it is *L. laccata, L. ohiensis,* or some adventitious species like *Paxillus involutus* (Moser 1967). The mycelia keep forming fruiting bodies even during the *Funaria*-stage, i.e., before the first *Nothofagus* seedlings appear (Moser 1949).

(3) The *Mortierella* group of soil fungi on the rhizoplane goes down to nearly half the population density 2 years after the fire, but reaches almost normal density again 7 years after the fire, as soon as pH and facultative ectomycorrhizae have been reestablished.

Table 1.—*Macromycete fructifications and root inhabiting fungi isolated from the soils of a humid zone where* Nothofagus dombeyi-Austrocedrus chilensis *forests were observed in climax and in regrowth stages after destruction by fire (Singer 1964–66, unpublished; Piterbarg 1966)*

Characteristics and composition of forest substrate	Stages of Forest Development				
	Climax[1]	Funaria[2]		Bryum[3]	New Forest[4]
Soil:					
Organic matter percent	26.6	8.4		14.0	20-28
pH	5.6	7		5.4	5.5
Root-inhabiting fungi[5]					Near
Mortierella sp. & *Mucor ramannianus*	46.1%	25.8%		43.6%	climax value
Phoma-Conothyrium-Pestalotia group	2.8%	18.5%		18.6%	Do.
Cylindrocarpon-Fusarium	4.1%	8.0%		4.1%	Do.
Macromycete fructifications	See list in text	12 mo. (Fall)	18 mo. (Spring)	24 mo. (Fall)	Spring and Fall	Fall
Galactinia ecinospora		0	0	+	0	0
G. nothofageti		0	++	0	0	0
G. petersii		+	0	0	0	0
Helotium citrinum		0	0	0	+++	+++
Sowerbyella thaxteri		0	0	0	+++	0
Pezizaceae sp.		0	0	0	+++	0
Dasyscyphus pygmaeus		0	0	0	++	0
Cotylidia undulata		++	0	+++	++	0
Schizphyllum commune		0	0	0	++	0
Pycnoporus cinnabarinus		0	0	0	++	0

Symbols: +, ++, +++ = relative abundance of species 0 = not evident

[1]Intact forest: *Nothofagus dombeyi* and *Austrocedrus chilensis* (mature trees and seedlings) abundant; also *Weinmannia* and *Myrceugenella* species present

[2] 1-2 years after total destruction by fire: ample *Funaria* cover; burned humus and ashes between

[3] 6-7 years after fire: young *Nothofagus* plants and seedlings established; a few *Weinmannia*, no *Austrocedrus* present; erosion rather strong

[4] 20 years after fire: dense, pure *Nothofagus dombeyi* woods established, *Myrtacea* and *Weinmannia* beginning or established in ravines, no *Austrocedrus* present.

[5] Isolates from roots of *N. dombeyi* seedlings.

Table 1—Continued

Macromycete fructification	See list in text	12 mo. (Fall)	18 mo. (Spring)	24 mo. (Fall)	Spring and Fall	Fall
Coriolus fernandezianus		0	0	0	+++	++
Dacryopinax spathularia		0	0	0	++	++
Polyporus melanopus		0	0	0	+	+
Clitocybe angustissima		0	0	0	++	++
Armillariella sparrei		0	0	0	+	+
Mycena parabolicformis		++	0	++	++	0
Mycena alcalina		0	0	0	+	0
Melanoleuca luteolosperma		0	0	0	+	+
Leucoagaricus atrofibrillosus		0	0	+	0	+
Coprinus atropides		++	++	0	0	0
C. carbonicola		0	0	+	++	0
C. auricomus		0	0	+	0	0
C. boudieri		0	0	0	0	0
C. subrenisporus		0	0	0	++	0
C. atramentarius		0	0	++	+	0
Psathyrella pennata		0	0	0	0	0
P. tristis		0	0	0	++	0
P. prona		0	0	0	++	0
Conocybe siennophylla var.		0	0	0	++	0
Conocybe tetraspora		0	0	0	++	0
Naematoloma sublateritium		0	0	0	+++	++
Psilocybe ecbola		0	0	0	+	0
Pholiota carbonaria		+++	+++	+++	++	0
Pholiota microcarpa		+	0	+	0	+
Pachylepyrium funariophilum		+++	0	0	0	0
Galerina berteroana		+++	0	+++	0	0
Galerina montivaga		0	0	+++	0	0
Alnicola amarescens		0	0	0	+	0
Tubaria furfuracea var.		0	0	+++	+++	+++
Facultative mycorrhiza forming species						
Laccaria tetraspora		+++	+++	++	+++	++
Pioneer obligatory sp. of mycorrhizal fungi:						
Inocybe fuscocinnamomea		0	0	0	Extremely scarce	++
Cortinarius sp. (M 3991)		0	0	0	Do.	?
Paxillus boletinoides						
Other mycorrhizal species:		0	0	0	Do.	++ more numerous than in climax
					0	

Symbols: +, ++, +++ = relative abundance of species 0 = not evident

(4) The adult forest resulting after 20 and even 30 years is an almost pure *Nothofagus* forest. There is no trace of the reappearance of *Austrocedrus*. This makes us suspect that in the wetter (western) zone of the *N. dombeyi-A. chilensis* association, the

existence of a limited stand without the conifer element indicates the destruction of an old climax forest, most probably by fire. In all such pure *Nothofagus* stands examined by us, we have been able to find carbon particles in the soil. It might even be possible that under certain climatic conditions such accidents may eventually contribute to the gradual restriction and retreat of *Austrocedrus* from ectotroph forests of Patagonia. This is a process favored by human activity since *Austrocedrus* is the most valuable timber produced and exploited in this type of forest. However, given a series of drier than normal years and favorable light conditions, *Austrocedrus* would gradually become the numerical dominant element.

In an area where *Nothofagus* had been eliminated completely, tree and fungal species were also identified (see table 2). In anectotrophic forests, the number of widespread to subcosmopolitan fungal species are considerably higher than that of species restricted to the *Nothofagus* region. However, in ectotroph-dominated forests, endemic species are more numerous than widespread ones (Singer 1964, Singer and Moser 1965).

In another study, twenty-five 3m x 3m plots at distances of 3m were marked off in a region connecting areas dominated by *N. dombeyi* and *A. chilensis* dominant areas. This region was somewhat off climax because of occasional wood cutting and the entrance of cattle. The fructifications in each square were identified in early and late fall (tables 3 and 4, starting on 211) providing quantitative data on the *N. dombeyi-A. chilensis* association untouched by fire.

This entire study shows that fungi are as important as the phanerogams in the characterization of the forest type. Since we were dealing with fruiting bodies rather than thalli as a whole, the data are more characteristic of certain floral aspects, such as the spring, early fall, and late fall fructification periods, than of the total biomass of soil-inhabiting mycelia present. The percentage of mycorrhiza-forming fungi among the total fungi is a function of edaphic-climatic conditions and of the number of ectotroph tree species in a community (Singer and Moser 1965). As long as the same fructification aspects are considered, the percentage of these fungi (whether computed by species or by individuals) remains constant for a given association.

Table 2.—*Ecosystem of* Austrocedrus chilensis-Nothofagus dombeyi *areas after the ectomycorrhizal tree dominant,* Nothofagus, *had been eliminated (1965)*

Trees in 12.5 x 2 m plots	Macromycetes	
	Humicolous and Litter Species (62%)	Lignicolous Agaric Species (38%)
Area I		
Austrocedrus chilensis++	*Camarophyllus pratensis*+	*Armillariella sparrei*+++
Lomatia hirsuta++	*Hygrocybe marchii*++	*Panellus longinquus*++
Myrceugenella apiculata++	*Hygrocybe sciophana*+	*Mycena austrororida*+
Flotovia sp.+	*Clitocybe patagonica*++	*Bolbitius aleuriatus*+
	Marasmiellus nothofagineus++	*Naematoloma sublateritium*+++
	Marasmius lomatiae++	*Simocybe olivaceonana*+
	Mycena albogrisea++	*Galerina austroandina*
	Mycena amygdalina+	*Pleurotellus hypnophilus*+
	Mycena microleuca+++	
	Mycena mirata+++	
	Mycena pura+	
	Psathyrella patagonica+	
	Rhodophyllus sp.+	
	Clavulinopsis pulchra++	
	Clavulina cristata++	
	Clavulina rugosa+	
Area II		
Austrocedrus chilensis (seedlings)+	*Camarophyllus pratensis*+++	*Armillariella sparrei*+
Lomatia hirsuta (seedlings)+	*Hygrocybe sciophana*+	*Mycena austrororida*++
Myrceugenella apiculata++	*Marasmius lomatiae*++	*Naematoloma sublateritium*+
Flotovia sp.+	*Mycena amygdalina*+	*Crepidotus sphaerosporus*+
	Mycena microleuca+++	"*Cyphella*" sp.+
	Mycena mirata+++	
	Mycena pura+	
	Psilocybe mesospora+	
	Rhodophyllus chloropolius+	
	Rhodophyllus dysthales+	
	Clavulinopsis pulchra++	
Outside Areas		
	Hygrocybe conica++	*Hohenbuehelia patagonica*+
	Lepista fibrosissima++	*Pluteus diptychocystis*++
	Coprinus comatus++	*Descolea recedens*+
		Phaeomarasmius myrceugenellae+
		Crepidotus applanatus++
		Morchella sp.
		Alnicola amarescens

Symbols: +, ++, +++ = relative abundance of species

Table 3.—*Early fall macromycetes in a zone (somewhat off climax) connecting* Austrocedrus chilensis *and* Nothofagus dombeyi-*dominant areas (1963)*

Fungi	Experimental plot number																									Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
SOIL & LITTER (37.2%)																										
Gerronema fibula	0	8	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9
Melanoleuca luteolosperma	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2
Collybia subhybrida	0	0	2	10	0	0	0	0	0	0	0	0	0	0	0	2	0	0	9	0	0	0	0	0	0	23
Fayodia striatula	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	4
Hydropus lipocystis	0	0	4	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	4	0	0	0	9
Mycena pura	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Mycena microleuca	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	10	6	0	0	0	0	0	19
Mycena albogrisea	0	0	12	0	0	1	0	0	0	3	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	18
Leucoagaricus atrofibrillosus	0	1	1	0	0	10	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	15
Lepiota cristata	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0.5	0	0	0	0	0	0	1	0	6.5
Psathyrella subandina	0	0.5	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.5
Conocybe cryptocystis	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	4
Galerina inconspicua	0	0	0	0	0	0	0	2	1	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	8
Tubaria furfuracea	1	1	1	0	5	7	6	0	0	0	2	0	2	17	4	3	1	2	0	0	0	3	0	0	0	55
Rhodocybe himantiigena	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Rhodocybe caelata	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	7	0	0	0	0	0	0	0	0	9
Rhodophyllus fuscifolius	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
LIGNICOLOUS AGARICS (54.4%)																										
Clitocybe pleurotus	0	29	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	30
Clitocybula mellea	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Panellus longinquus	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
Campanella dendrophora	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	48	0	48
Mycena pseudovulgaris	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	12
Mycena pseudoalnicola	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
Melanotus gayi	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Pholiota microcarpa	0	4	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7
Simocybe curvipes	0	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30
Galerina patagonica	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Crepidotus sphaerosporus	7	0	7	0	0	0	0	0	6	50	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	82

Table 3.—*Early fall macromycetes in a zone (somewhat off climax) connecting* Austrocedrus chilensis *and* Nothofagus dombeyi-*dominant areas (1963)*—Continued

Fungi	Experimental plot number																									Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
Crepidotus nephrodes	0	0	0	0	0	0	0	35	0	0	0	0	0	0	0	0	0	0	0	0	2	5	0	0	0	42
Crepidotus applanatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
Crepidotus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	5
FACULTATIVE MYCORRHIZA-FORMING (5.8%)																										
Laccaria tetraspora	9	9	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	25
Clavariella patagonica	0	0	3	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
OBLIGATORY MYCORRHIZA-FORMING (2.6%)																										
Cortinarius sp. M 3326	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	3	0	0	0	0	0	0	4
Cortinarius sp. M 3321	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	9

Table 4.—*Late fall macromycetes in a zone (somewhat off climax) connecting* Austrocedrus chilensis *and* Nothofagus dombeyi-*dominant areas (1964)*

SOIL & LITTER (51.2%)																										
Hygrocybe singeri	3.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.5
Lyophyllum langei	15	0	4	0	0	0	0	4	3	0	1	0	0	3	1	0	1	0	2	3	0	0	0	0	2	39
Clitocybe angustissima	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	3
Clitocybe obscuratipes	0	6	0	0	0	0	0	0	0	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	10
Gerronema minutum	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	2
Ripartites tricholoma	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2
Melanoleuca melanosarx	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1.5
Melanoleuca luteolosperma	0	0	1	1	1	0	1	20	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	28
Macrocystidia cucumis	0	0	0	0	3	0	1	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0	9
Collybia subhybrida	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	1	0	0	0	0	0	1	0	0	5
Collybia cf. *butyracea*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Campanella dendrophora	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	5	0	0	17

Hydropus lipocystis	6	0	1	0	2	0	6	0	1	4	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	21
Mycena pura	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	3
Mycena microleuca	126	0	0	0	1	1	0	31	3	8	0	11	0	0	0	0	0	2	1	37	6	43	2	27	100	0	33	436
Mycena haematopoda	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Mycena austroavenacea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	15	0	17	0	0	0	0	0	0	0	33
Mycena phyllogena	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Mycena sp. (ignota ined.)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
Leucoagaricus atrofibrillosus	0.5	0	1	0	0	0	1	2	3	0	0	0	0	3	0	0	0	1	0	4	0	2	1	1	1	0		20.5
Leucoagaricus erthrellus	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0		6
Melanophyllum echinatum	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		3
Cystolepiota australis	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		1
Lepiota neuquenensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0		1
Lepiota crystata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2		4
Coprinus disseminatus	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		20
Psathyrella polycystidiata	0	0	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0.5
Psathyrella dactylocystis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	0	0	0	0	0	0	0		5
Pholiotina verrucispora	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		3
Descolea recedens	0	0	2	0	0	2	1	0	0	2	0	0	0	20	3.5	0	0	0	0	1	13	0	0	0	0	0		44.5
Psilocybe araucana	0	0	0	0	0	5.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		5.5
Pholiota baeosperma	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0		1
Psilocybe fuegiana	0	7	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0		7
Galerina inconspicua	0	17	0	19	3	30	5	2	2	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0		89
Galerina patagonica	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		5
Tubaria furfuracea	0	0	4	3	0	0	1	2	0	3	0	0	0	0	0	1	0	1	0	19	0	0	4	17	4	2		61
Rhodocybe himantiigena	0	1	0	0	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		16
Rhodocybe caelata	0	0	0	0	0	7	0	0	1	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0		12
Rhodophyllus chloropolius	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		1
Rhodophyllus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0		6
Clavulina cristata	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		9
Hysterangium separabile	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0		1

Table 4.—*Late fall macromycetes in a zone (somewhat off climax) connecting* Austrocedrus chilensis *and* Nothofagus dombeyi-*dominant areas (1964)*—Continued

Fungi	Experimental plot number																									Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
LIGNICOLOUS AGARICS (12.7%)																										
Clitocybe subleptoloma	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Mycena haematopoda	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	2	23
Mycena galericulata	0	0	0	5	0	0	0	0	1	0	3	0	0	0	1	2	0	0	0	0	0	0	0	2	0	14
Pluteus jaffuelii	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Pluteus spegazzinianus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
Psathyrella polycystidiata	0	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0	1	8	8	0	17.5
Bolbitius aleuriatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
Naematoloma sublateritium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	2	0	0	0	11	15
Melanotus patagonicus	0	0	0	0	0	6	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10
Pholiota baeosperma	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Tubaria furfuracea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	6	0	16
Crepidotus sphaerosporus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	12	0	8	30	0	24	0	30	0	116
Crepidotus nephrodes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	12
DUNG AGARICS (0.1%)																										
Panaeolus campanulatus	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Stropharia semiglobata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
FACULTATIVE MYCORRHIZA-FORMING (16%)																										
Laccaria tetraspora	5	33	9	0	0	3	8	0	0	14	3	0	28	19	15	0	0	0	0	0	1	2	0	3	5	148
Clavariella patagonica	3	0	0	0	0	0	0	0	0	0	0	150	0	1	0	0	0	0	0	0	0	0	0	0	0	154
OBLIGATORY MYCORRHIZA-FORMING (21%)																										
Porpoloma sejunctum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	2
Inocybe sp. A, Aa	74	54	25	0	0	0	0	0	0	0	0	1	0	0	13	0	4	0	0	3	13	6	2	1	67	263
Inocybe (6 sp.)	0	9	0	3	0	0	0	0	0	0	0	1	0	2	2	6	5	18	9	6	8	7	1	2	2	81
Cortinarius sp. C	0.5	0	0	0	0	0	0	0	0	0	0	0	0	3	5	1	9	4	0	0	1	3	1	0	0	18.5
Cortinarius sp. A/B, D/S (17 sp.)	3.5	0	1	0	0	0	0	0	1	0	1	0	3	3	1	4	3	4	3	1	0	0	1	2	1	32.5
Paxillus defibulatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	3	0	4

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Growth Promotion of Slowly Growing Mycorrhizal Basidiomycetes in Submerged Culture

V. Šašek and V. Musílek

The growth of mycorrhizal basidiomycetes in the laboratory is generally slower than that of nonmycorrhizal basidiomycetes. This is of interest not only from a theoretical point of view but also from a practical one, especially if some of these organisms produce biologically active substances such as antibiotics. Antibiotics from the mycorrhizal fungi *Rhizopogon roseolus* and *Tricholoma saponaceum* were detected by Šašek and Musílek (1968), but the very slow growth of the fungi and the length of time necessary for antibiotic production precluded the exploitation of these organisms in commercial antibiotic production. Consequently, *R. roseolus* (Corda in Sturm) Th. M. Fr. was chosen as a model organism for more detailed studies of the factors influencing growth and antibiotic production in mycorrhizal basidiomycetes.

In our experiments, substances known to promote growth in other fungi, such as vitamins (especially B vitamins), amino acids, yeast extract, corn steep, and casein hydrolysate were added to the synthetic medium of Norkrans (1950). The fungus was transferred into the medium and cultivated on a reciprocal shaker at 20° C. Coconut milk and a combination of kinetin and hetero auxin—factors known to be very effective on plant tissue cultures —were also tested. Some of these materials caused increases in dry weight, but 3–5 weeks were still required for maximum growth.

At the beginning of the rapid growth phase, irregular, small flakes of mycelia as well as the usual mycelial pellets are observed. When an inoculum in this phase was used, the new culture reached the growth maximum several days earlier than the 4-week-old cultures previously used. Flake formation was also more rapid. Repeated transfers of progressively younger cultures produced maximum growth after about 9–10 days of cultivation (fig. 1). Furthermore, the macroscopic appearance of the cultures was also changed. The mycelial pellets typical of old cultures were replaced by the more dispersed flake-like mycelia (fig. 2).

Since the dispersed "activated" inoculum contained 8-10 times less mycelia than the original, the increase in growth rate of *R. roseolus* cannot be attributed to the presence of more starter centers of growth. Better growth was obtained with 32-day-old inoculum if the mycelia were dispersed by homogenation, but this increase was not comparable to that evoked by "activated" inoculum.

The effect of such "activated" inoculum is probably not general in basidiomycete cultures. When a fast-growing, wood-destroying basidiomycete [*Oudemansiella mucida* (Schr.) Pat.] was treated in the same way, corresponding results were not obtained. For example, maximum growth was obtained with this organism in ap-

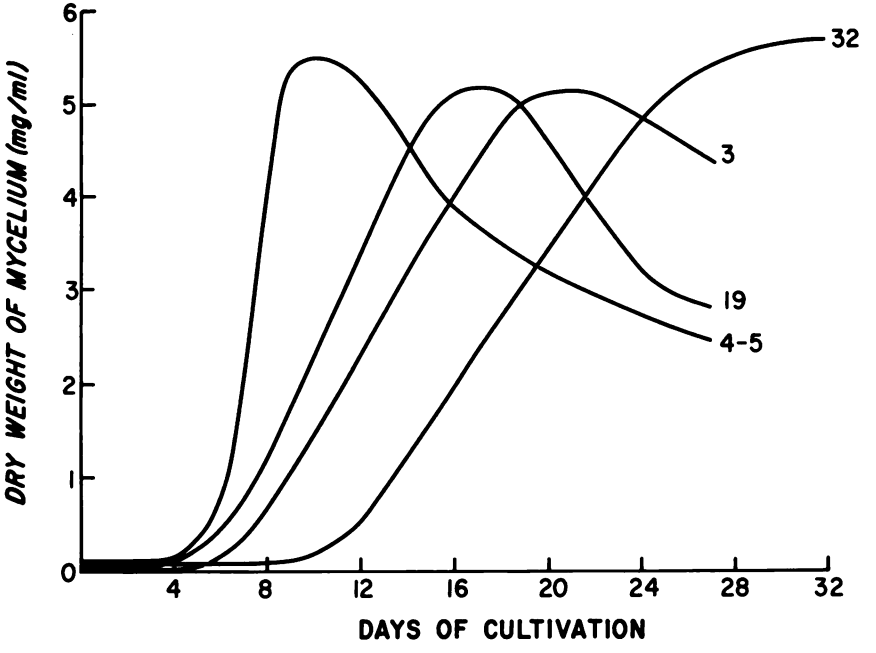

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Figure 1.—The effect of gradually rejuvenated inoculum on the growth of *R. roseolus*. The number by each curve refers to the age of the inoculum in days.

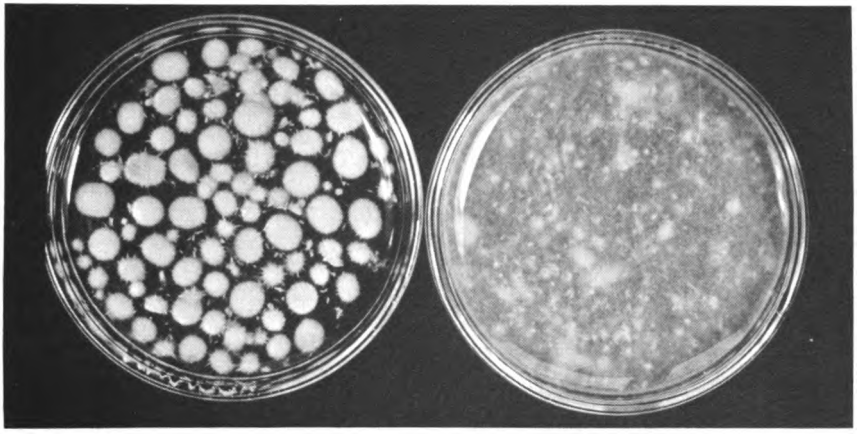

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Figure 2.—The macroscopic morphology of the original pellet-like culture and the more dispersed culture obtained from "activated" inoculum. Both cultures were photographed when they reached maximum growth (i.e., the original culture after 32 days of cultivation, the other one after 10 days of cultivation). For photography, the cultures were transferred from shaken flasks into Petri dishes. 4 cm diameter.

proximately the same time when 2-, 5-, or 9-day-old cultures were used as inocula.

Estimation of the chemical composition of mycelia from "activated" cultures of *R. roseolus* during cultivation showed that the percentage of protein and deoxyribonucleic acid content was changed only a little. However, it is of interest that a peak in ribonucleic acid content was detected after 4–5 days of cultivation when the culture was best suited as inoculum. The more detailed biochemical characterization during cultivation is under further study.

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Separate No. FS-301

Production of Antibiotics by Certain Mycorrhizal Fungi

George N. Krywolap

The symbiotic nature of the mycorrhizal association has never been clearly defined. The host plant has been shown to provide the fungus with carbohydrates, vitamins, and unknown factors for growth (Harley 1952). Several theories have been advanced to explain the benefits of the fungal component to the host plant. Most suggest that the mycorrhizal seedling is better able to compete with the soil microflora for the poorly available nutrients of forest soils (Wilde 1958, Zak 1964).

An additional competitive advantage, that of antibiotic production by mycorrhizal fungi, also has been demonstrated by a number of workers (Santoro and Casida 1962; Krywolap and Casida 1964; Šašek and Musílek 1967, 1968; Wilkins and Partridge 1950). Krywolap, Grand and Casida (1964) have shown that an antibacterial antibiotic isolated from pure cultures of *Cenococcum graniforme* was present in nature in the mycorrhizae, roots, and needles of red and white pines. In another study, Šašek and Musílek (1968) showed that, under laboratory conditions, the growth of certain parasitic species could be retarded by mycorrhizal fungi. Thus, the production of antibiotics by mycorrhizal fungi becomes of interest: their elaboration by the fungi during mycorrhizal association could aid a tree seedling in its competition with the soil microflora for the poorly available nutrients of the soil as well as ward off certain potential plant pathogens.

In the present study,[1] extracts of vegetative mycelia and filtrates of twenty-six cultures of mycorrhizal fungi were studied for their antibiotic content. Cultures of *Amanita caesaria, A muscaria, A. phalloides, A. pantherina, A. rubescens, Boletus bicolor, B. luteus* and *Rhizopogan roseolus* were obtained from Dr. E. Hacskaylo of the Forest Physiology Laboratory, U. S. Department of Agriculture, and cultures of *A. mappa, A. silvicola, B. communis, B. laki, B. variegatus, Cantharellus floccosus, Clitocybe piceina, Clitopilus prunulus, Corticium bicolor, Ithyphallus ravenelli, Laccaria laccata, Lactarius chrysorheus, L. deliciosus. Lepiota capaestris, Pisolithus tinctorius, Russula emetica, Suillus subolivaceous* and *Tricholoma personatum* were obtained from Drs. B. Zak and J. M. Trappe of the Forestry Science Laboratory, Pacific Northwest Forest and Range Experimental Station of the U. S. Department of Agriculture.

Mycorrhizal fungi for antibiotic studies were grown by the "bot-

[1] This research was supported in part by U.S. Public Health Service Grant FR 5317-07.

tlebead" technique (Santoro and Casida 1959) in Fernbach flasks on amended Hoagland's medium (Santoro and Casida 1962) for 14 days at 25° C. Solvent extractions of vegetative mycelia and antibiotic assays of culture filtrates and solvent extracts were carried out in a manner similar to that described in an earlier paper (Krywolap and Casida 1964).

The results obtained are summarized as follows:

1. The antibiotic activity against *Bacillus cereus* and *Escherichia coli* was present in culture filtrates of *Boletus laki, B. variegatus, B. communis, Corticium bicolor, Clitocybe piceina* and *S. subolivaceous.*
2. Culture filtrates of *A. muscaria, A. rubescens, A. caesaria, A. phalloides, B. bicolor, I. ravenelli, Laccaria laccata, Lactarius chrysorheus, Lepiota capaestris, P. tinctorius* and *T. personatum* showed activity only against the gram negative organisms tested—*E. coli* and *Shigella sonnei.*
3. Culture filtrates of *A. mappa* were active against *Bacillus cereus* and *Arthrobacter globiformis* but not against *E. coli* or *Sh. sonnei.*
4. Acetone and chloroform extracts of moist mycelia of *A. muscaria, Boletus bicolor, A. pantherina, B. luteus. Lactarius deliciosus* and *R. emetica* showed activity against *Bacillus cereus* and *E. coli.*
5. No activity against *B. cereus* and *E. coli* was present in culture filtrates or acetone and chloroform extracts of *A. silvicola, Rhizopogon roseolus, Cantharellus floccosus* and *Clitopilus prunulus.*
6. Except for the antibiotic produced by *Boletus communis,* the antibiotic potencies observed for the culture filtrates and solvent extracts of the tested fungi were low and poorly reproducible.

This study confirms the findings of Santoro and Casida (1962) for the elaboration of antibiotics by *B. luteus, B. bicolor, A. caesaria* and *A. muscaria* grown on amended Hoagland's medium. We failed, however, to demonstrate antibiotic activity either in culture filtrates or solvent extracts of *C. prunulus* and *R. roseolus,* although positive results were reported by Šašek and Musílek (1967). In the same paper, activity was shown in culture filtrates and/or mycelial solvent extracts of *A. pantherina, A. muscaria, B. luteus, B. variegatus* and *Lactarius deliciosus* which were reported to be negative. Similarly, activity was shown to be present in *T. personatum* which was reported to be negative by Wilkins and Partridge (1950).

These discrepancies can, in part, be attributed to the variations in methodology used in growing fungi for antibiotic production. Composition and initial pH of the medium, duration, and temperature of incubation, as well as assay procedures, have been shown to influence the ability as well as consistency of antibiotic production by certain mycorrhizal fungi (Santoro 1961).

Variations in antibiotic production among various strains of a single species of mycorrhizal fungus have been reported by Wilkins and Partridge (1950). Out of 266 common species examined and then compared to the findings of Hervey (1947), 40 percent were not in agreement. Thus, it would appear that, besides the influence

of the environment on antibiotic production by mycorrhizal fungi, ability to produce antibiotics is a function of an individual strain rather than that of a species, and this possibility should be kept in mind when screening mycorrhizal fungi for antibiotics.

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Transport of C^{14}-labeled Substances in Mycelial Strands of *Thelephora terrestris*

C. P. P. Reid

There has been more and more interest expressed in how nutrients are cycled in an ecosystem, and in particular, how cycling might occur in the rhizosphere. There are many examples where substances are transferred between root systems by means of root grafts (Graham and Bormann 1966). It is also well documented that plant roots exude a variety of endogenous substances (Rovira 1962, Slankis, *et al.* 1964. These substances are not only potentially available for transfer to another root system, but have actually been shown in some instances to be transferred from root to root. Several years ago, investigations were conducted in Duke Forest where the radioisotopes Ca^{45} and P^{32} were applied to maple stumps in a mixed hardwood stand (Woods and Brock 1964). These isotopes were transferred to 19 different species of woody plants by way of the rhizosphere. At that time, mycorrhizal structures were implicated as possibly contributing to the rapid exchange of the radioisotopes.

Investigations were therefore conducted with mycorrhizae under laboratory conditions to test the hypothesis that there is a potential for interplant transfer of materials by way of shared mycorrhizal fungi. One part of these investigations, the movement of C^{14}-labeled substances in mycelial strands of *Thelephora terrestris* (Ehrh.) Fr., is reported here.

Loblolly pine seedlings (*Pinus taeda* L.) were grown in the presence of *T. terrestris* in axenic culture for 7-8 months. In addition to having abundant mycorrhizae present after this time (fig. 1), mycelia were aggregated into strands easily visible between the substrate and inside surface of the glass container. Because of this unique type of growth, the container could be broken and removed without disturbing the substrate, strands, or roots.

This presented an ideal way to investigate the movement of substances in mycelial strands which were still attached to mycorrhizal roots. After removal of the glass container, the plant was placed in a chamber with the foliage atmospherically isolated from the root system (fig. 2).

The strands were pulled away from the substrate without severing their connections with the roots. Strands of varying length and roots were separated from the substrate and supported to prevent contact with the container. To determine transport of organic substances from the root system to the strand, C^{14}-labeled sucrose or glucose was applied to the apical foliage of the seedling. Samples of roots, strands, and vermiculite substrate were removed periodically, digested, and counted for C^{14} in liquid scintillation. Previous studies, in which labeled sugars were applied to the plant in the same manner, indicated that C^{14} readily moved into the root

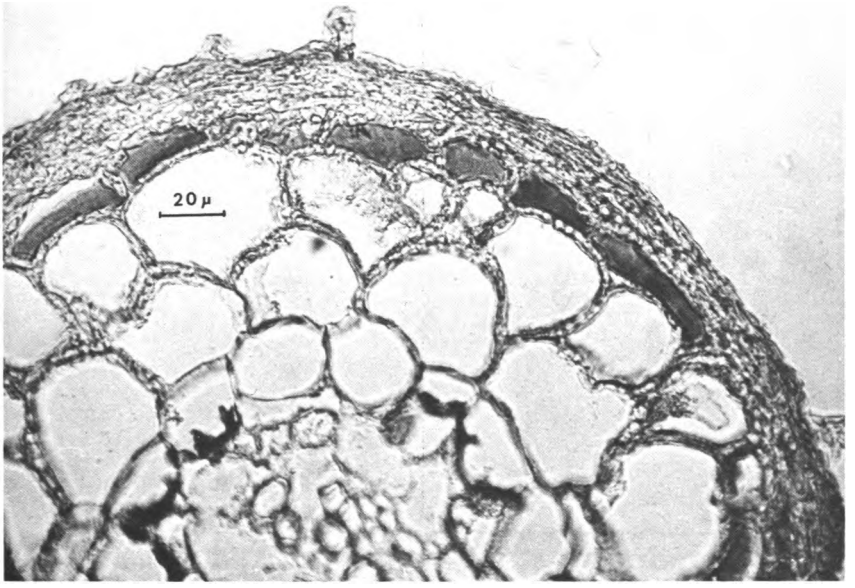

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Figure 1.—Cross section of *Pinus taeda/Thelephora terrestris* mycorrhiza formed in axenic culture.

systems (fig. 3). In some cases, an additional mycorrhizal seedling was also placed in the chamber. This plant was not treated with isotope but was sampled to serve as a control in the event of atmospheric transfer of C^{14}. Containers of NaOH were placed in the chamber to trap any $C^{14}O_2$ respired in the root zone.

The reciprocal flow of C^{14}-labeled substances from the external strand to the seedling was investigated by immersing the tips of mycelial strands in 30-40 μl of 0.05M sugar solution containing C^{14}-labeled sucrose or glucose. Samples of roots, needles, and distant mycelium were then collected to determine C^{14} content. In addition to liquid scintillation counting, autoradiographs were made of the seedlings.

Table 1 presents the results from applying glucose-C^{14} to the foliage. Significant activity was detected in the root samples and two mycelial strands after 48 hours. Strand No. 1, with highly significant activity in all samples after 48 hours was more than 12 cm in length at termination of the experiment. Strand No. 2 was 3 cm in length and showed some activity after 48 hours. Autoradiographs showed C^{14} well distributed in the root system, as expected, and in the mycelial strands. No C^{14} was detected in the vermiculite samples.

When C^{14}-labeled sucrose was applied to the foliage, significant quantities of C^{14} were found in the root samples as well as the two strands sampled (table 2). One strand showed activity after 120 hours and the other, after 144 hours. In this trial, the untreated mycorrhizal seedling was present in the chamber and was also

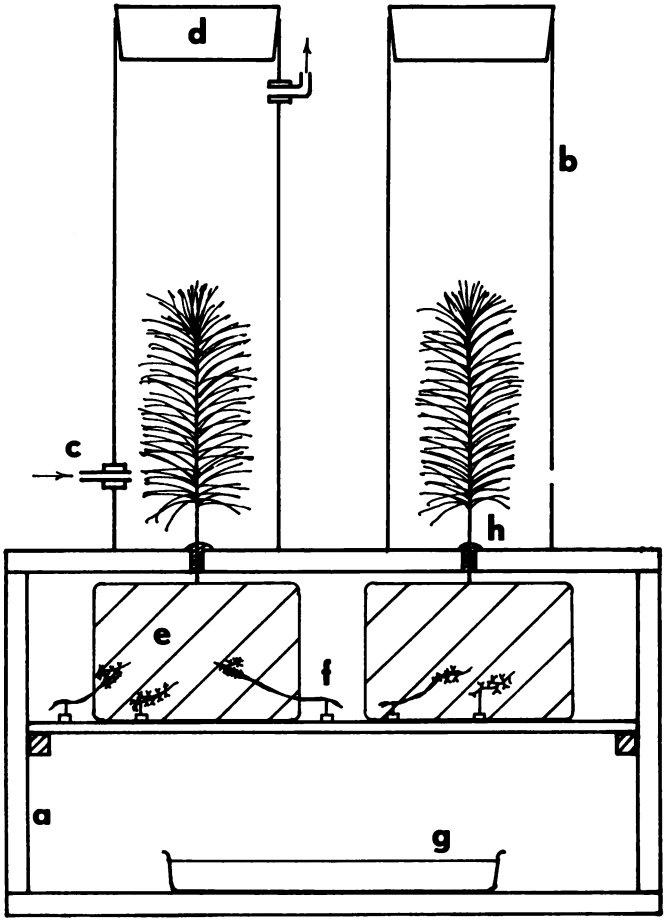

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Figure 2.—Schematic cross section of chamber used for introducing C^{14}-labeled compounds to pine seedlings with mycelial strands of *Thelephora terrestris*. a. Plywood container. b. Plastic cylinder. c. Inlet for air circulation. d. Rubber stopper. e. Substrate mass with roots and mycelium. f. Separated mycelial strand. g. Free water surface for humidification. h. Sealant around stem.

sampled. The absence of significant activity indicated there was no atmospheric transfer of $C^{14}O_2$.

When glucose-C^{14} was applied to a mycelial strand to determine transport of C^{14}-labeled substances to the root system, C^{14} was detected in the roots and also in the needles after 24 hours (table 3). The seedling was autoradiographed with the treated strand intact (fig. 4). The treated strand was heavily labeled as expected. The root directly attached to the strand was labeled throughout its length. The apical buds were labeled slightly more than the foliage. When sucrose-C^{14} was applied to a separated strand, C^{14}

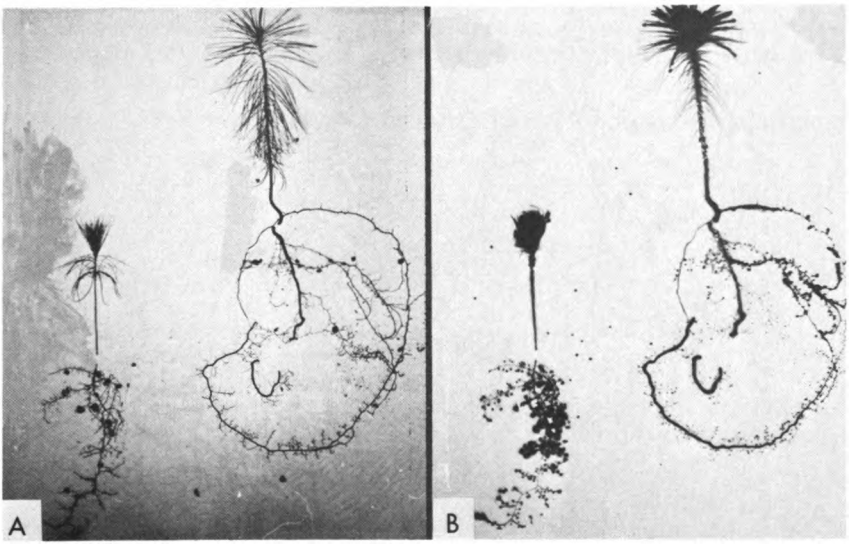

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Figure 3.—Distribution of C^{14} in loblolly pine seedlings after introduction of C^{14}-labeled glucose to the apical foliage: A. Seedlings treated with radioisotope, B. Autoradiographs of same seedlings.

Table 1.—*C^{14} activity in mycelial strands, roots, and vermiculite after glucose-C^{14} introduction to pine foliage*

Source of sample	Activity (counts/min) after isotope introduction Hours 24	48	72	96	120
Mycelial strand					
1	0	35*	123*	85*	38*
2	0	11**	5	14*	
3	0	0.1	0		
4	0.1	0	4	0	
5	0	0	7	2	
Root	5	21*	125*	117*	82*
Vermiculite					
1	0	0	0	0	0
2	0	0	0	0	0

*=count rate significant at 1% level.
**=count rate significant at 5% level.

was detected in the roots after 120 hours (table 4). The autoradiograph of the seedling showed a short segment of root labeled with isotope; however, none was transported to the needles.

One additional trial was conducted where a mycelial strand of one seedling was physically wrapped around the mycorrhizal roots of another seedling. Glucose-C^{14} was then applied to one seedling (donor), and the second seedling (recipient) was sampled. Table 5

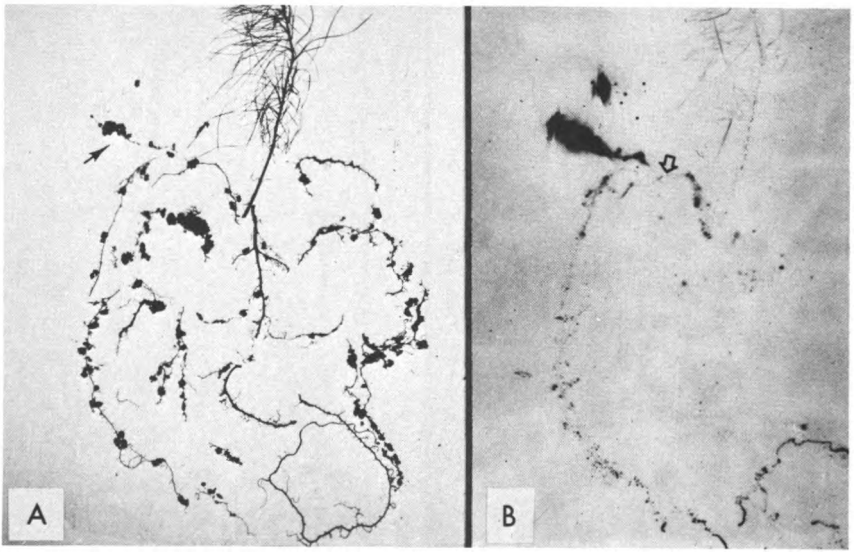

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Figure 4.—Transfer of C^{14} from strand of *Thelephora terrestris* to loblolly pine seedling: A. Seedling and strand (arrow) treated with glucose-C^{14}, B. Autoradiograph of same seedling and strand with arrow showing connection between strand and root.

Table 2.—*C^{14} activity in mycelial strands, roots and needles at successive intervals after sucrose-C^{14} introduction to pine foliage*

Source of sample	Activity (counts/min) after isotope introduction Hours									
	24	48	72	96	120	144	159	192	216	242
	C^{14} treated seedling									
Root	11**	13*	69*	45*	84*	219*	159*	61*	311*	60*
Strands										
1	0	0	1	3	18*	6	23*	13*	9	9
2	2	2	0	3	3	26*	22*	18*	43*	9
	Untreated seedling									
Root	0	0	0	0	0	3				
Strands										
1	2	0	0	0	2	0				
2	0	3	0	0	0	1				

*=count rate significant at 1% level.
**=count rate significant at 5% level.

shows the high level of activity in the roots of the donor and the subsequent transfer of C^{14} to the recipient. Significant activity was detected in the recipient root and foliage after 48 hours.

The demonstration of the movement of C^{14}-labeled organic substances in the mycelial strands of *T. terrestris* as shown by the

Table 3.—*C^{14} activity in roots, needles, and mycelium at successive intervals after glucose–C^{14} introduction to mycelial strand of* Thelephora terrestris

Source of sample	Activity (counts/min) after isotope introduction Hours 24	48	96	120
Root 1	40*	30*	34*	54*
Root 2	9	15*	10**	58*
Mycelium	0	0	0	0
Needle	34*	123*	84*	77*
Strand base				241*

*=count rate significant at 1% level.
**=count rate significant at 5% level.

Table 4.—*C^{14} activity in roots, needles, and mycelial strands at successive intervals after sucrose–C^{14} introduction to mycelial strand of* Thelephora terrestris

Source of sample	Activity (counts/min) after isotope introduction Hours 24	48	72	96	120
Root	4	0	0	0	34*
Needle	4	5	5	0	6
Strand base	6040*	0	20*	169*	4
Strand (unattached)	0	2	0	0	0

*=count rate significant at 1% level.

Table 5.—*C^{14} activity in roots, needles, and mycelium at successive intervals after glucose–C^{14} introduction to pine foliage*

Source of sample	Activity (counts/min) after isotope introduction Hours 24	48	72	144	168
Root (donor)	919[1]*	1976*	818*	933*	1367*
Root (recipient)	5	16*	15*	9	9
Needle (recipient)	5	35*	16*	19*	41*
Mycelial strand (recipient)					226*

[1]*=count rate significant at 1% level.

data above would indicate that there is a potential for interroot transfer of substances between plants sharing the same mycorrhizal fungus. It is further believed that there are ecological circumstances where such transfer could occur under natural conditions.

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Mycorrhizae and Nutrient Cycling in the Tropics

N. M. Stark

The tall, lush rain forests along the Amazon convinced the early European settlers that the soils were the richest in the world. However, after cutting and burning the jungle, they soon found the land too sandy and unsuitable for agriculture. Today, these abandoned areas consist of poor second growth forests or degenerate rain forests (Went and Stark 1968), while a few meters away on uncleared land, climax trees of 30 m tall grow on the same podzolized soils.

The Brazilian soil is primarily sand, covered by a thin surface layer of organic material (1 to 3 cm deep) and litter (1 to 2 cm deep). The sand is tan to white, slightly coarse, and almost devoid of clay. Chemical analyses for extractable cations produced figures so low (70 to 210 μg/g) that it was difficult to believe that a rain forest once grew there. Compared to Hewitt's (1966) nutrient solution which is ideally suited for tomato growth, the poorest Brazilian soils are deficient in calcium, magnesium, sodium, and potassium.

In contrast, we found that the nutrient level of the litter ranged from 1,425 to 7000 μg/g of calcium, 950 to 3,150 μg/g of magnesium, 100 to 1,250 μg/g of sodium, and 500 to 2,500 μg/g of potassium. The average of the total content of ten biologically important elements in a gram of plant material is 4,415 μg/g, and in a gram of soil, 1,044 μg/g, or about one-fourth that found in the plant. If only extractable cations are considered, there are about 6 μe of elements available in 1 g of Brazilian sand. Since plants are known concentrators of nutrients, it is not fair to equate plant nutrients per gram to soil nutrients per gram.

Litter is known to release biologically important elements at the rate of 25,640 μe/m^2/day, and since the daily leaf fall alone delivers 27,180 μe/m^2/day, there is always an adequate supply of fresh nutrients available in the litter. We do not know how rapidly the soils release nutrients, but it is doubtful that they even approach this rate; their total content of biologically important elements is so low that the nutrient supply, if not replenished, would be exhausted in a few hundred years.

Although the large support roots of Brazilian climax trees grow deep into sand, the feeder roots do not; 90 percent of them are concentrated in the litter and the upper 3 to 10 cm of the soil. Microscopic examination of these feeder roots shows that a large number of them are infected with mycorrhizal fungi, and many of these, with endomycorrhizal fungi. Hyphae were also found in tissues of dead woody fruits, branches, and leaves, which make up the litter layer. In one case, I traced a hypha from a dead fruit into a living feeder root cell. This is a possible route of transport for nutrient materials.

The roots of poor second growth forest trees are not always mycorrhizal, but the feeder and support roots are usually spread throughout a sizeable soil mass. Recent measurements show that the second growth vegetation can concentrate calcium, phosphorus, nitrogen, and other elements to levels significantly higher (1 percent level) than can nearby climax vegetation on the same soils. But the cutting and burning of rain forest vegetation for crops released great quantities of nutrients from the organic matter leaving them soluble and easily leached and lost.

It is possible that in extremely poor soils, mycorrhizae are important in supplying nutrients directly from litter to living roots. However, much more study is needed. We expect to learn a great deal about the role of mycorrhizae in nutrient cycling by using radioactive tracers to follow the decomposition products of litter.

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Separate No. FS-304

Mycorrhizae in a Montane Pine Forest

F. W. Went

In the Amazon Rain Forest, Dr. Stark and I recently discovered that mycorrhiza is not just a bipartite (root-fungus) relationship, but that it is tripartite, with the fungus forming a link between organic soil litter on the one hand and roots on the other (Went and Stark 1968a, 1968b). This is the closing link of the mineral nutrient cycle, which has been generally assumed to exist in lush rain forests where soils are poor in minerals (Richards 1952). Dr. Stark has presented evidence of this. Most mycorrhizal systems are very complex with dozens of trees and higher plants involved; however, I want to discuss a very simple system: the lodgepole pine forest of Eastern Sierra, Nevada, which grows in areas with abundant sub-surface water but where the upper soil horizons are very dry all summer.

The lodgepole pine forest I studied is located in Little Valley, an isolated uninhabited valley at 1900 m altitude located just east of Lake Tahoe on 40 km due south of Reno. Part of it consists of a very dense stand of *Pinus murrayana,* the shade underneath being so dense that for many observations during day, a flashlight is necessary. There is no phanerogamic undergrowth, except for pine seedlings and saprophytes, such as pinedrop, *Pterospora andromedea.* The extensive die-back of the lower pine branches shows the degree to which the light intensity under the canopy remains below the compensation point; the pinedrop and pine seedlings both obviously must be fed by mycorrhizal fungi to survive.

Starting at the surface, the soil in this forest consists of a 5 cm layer of loose, undecayed litter (L), made up of dry needles, branches, and male and female cones from the pine canopy. The next 5 cm is consolidated decomposing litter, held together by a dense mycelial and rhizomorphic network, with no soil or sand particles. In the densest stands, there are practically no animals such as ants, nematodes, mites, or Collembola to move soil particles from the A horizon into the decomposing litter layer (H). The A horizon consists of the actual soil, mostly sand with pebbles and stones, interlaced with much humus. Pine roots occur mostly in the upper A layer, penetrating into the lower H layer. Rhizomorphs, both the feathery white ones and the black ones with dense sheaths surrounding hyphal bundles, are most abundant in the H layer, but extend into the A horizon and connect with mycorrhizae.

The microorganisms in the L and H layers are almost completely

[1] This work was supported by Grant GB 6681 from the National Science Foundation. Identification of the fungi was by Drs. J. A. von Arx, K. B. Raper, and E. W. Hesseltine.

restricted to fungi, with only a few hundred bacteria per gram of litter. In contrast, bacteria are associated with every soil particle in the A layer. In the L layer, only a few species of Dematiae (*Cladosporium macrocarpum* and *C. cladosporioides*) predominate. These grow on and in the 0–1 year old needles, apparently digesting the mesophyll cell contents.

For our investigations, the fungi of the H layer were isolated either by placing pieces of litter on nutrient agar or by inserting *in situ* branches, rhizomorphs, or roots into test tubes so that their tips just touched the agar of the slant. Usually just a single species of fungus started to grow from the litter, roots, or rhizomorphs, and those developing were in 90 percent of the cases: *Mucor hiemale, Mortierella* (?), and *Penicillium terrestre,* all three forms emerging from both litter, rhizomorphs, and roots. This is a good indication that these three fungi connect litter with roots, decomposing the cell wall materials and transferring the released nutrients by way of the rhizomorphs to the roots.

Another method was used to study these fungi. When roots, rhizomorphs, and litter particles were washed free and placed in a moist chamber without additional food in a cool laboratory (10°C day and 5°C, night), hyphae emerged from all litter. The cut ends of rhizomorphs sprout bundles of long hyphae, which pervade all surrounding space and secrete sugar droplets. On the ends of the mycorrhizal branches, crowns of hyphae or conidiophores are produced. These conidiophores resemble *Penicillium terrestre.* In each group of mycorrhizal branches, the mycelium or hyphae produced look so similar, that it seemed unlikely we were dealing with infections. This all shows that one does not need special media to bring out the effective fungi; they can proceed on their own. In this system, there is apparently no niche for bacteria, at least during summer.

The various litter and soil layers as described earlier were separated, and six pots were filled with each of these layers and placed in a cool greenhouse (20°C, day and 10°C, night). A number of seeds present in litter and soil germinated (see table); the light seeds of *Mimulus* and *Juncus* were found predominantly in the A horizon, whereas the 100 times heavier seeds of *Montia* were mostly in the L and H layers. There was only one mushroom species which developed in 4½ months time: *Sphaerobolus,* present in all pots with undecomposed litter, and in four out of six pots with decomposed litter, with only one-fifth the number of fruiting bodies. And none developed with the A horizon soil. All seedlings grew rapidly except those in pots with the undecomposed litter (L).

In conclusion, we can say that the simple ecosystem of the *Pinus murrayana* forest provides for a complete organic cycle with hardly any loopholes: 1) photosynthesis and production of needles, branches, and cones, 2) decomposition of cell contents by Dematiae, 3) decomposition of cell wall materials and lignin by *Mucor hiemale, Mortierella,* and *Penicillium terrestre,* 4) return of the released sugars and minerals by way of rhizomorphs and mycorrhizae to roots. In addition, the ecosystem contains enough seeds for a dense vegetation of herbs. In this almost closed ecosystem, not only are most minerals recycled but the photosynthates fixed in cell materials can also be reused by the pine roots, pine seedlings, and sapro-

phytes. This means translocation of photosynthates by gravity from tree crown to ground and by fungi from litter to roots may be a feature in this forest; translocation of minerals by this means, as suggested by Stark (these proceedings), producing a direct mineral cycle, is also very likely.

Table—*Total number of seedlings and fruiting bodies in soil layers from a* Pinus murrayana *forest*

Horizon from which soil was taken	Soil Description	Number of seedlings per 6 pots			Number of *Sphaerobolus* fruiting bodies per pot
		Montia perfoliata	*Mimulus primuloides*	*Juncus articulatus*	
L	Undecomposed litter	9	2	6	12.7±3.3
H	Decomposing litter	8	3	3	2.5±1.0
A_0	Sand with much humus	8	9	10	0
A_1	Sand with less humus	1	9	8	0

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Correction of Mycotrophic Deficiencies of Tree Nursery Stock Produced on Biocide-Treated Soils[1]

Jaya G. Iyer, Erkki Lipas, and Gordon Chesters

Some of the eradication treatments of nursery soils decrease significantly the survival potential of planting stock by imparting to trees undesirable properties such as low specific gravity tissue of high succulence, underdeveloped root systems of low absorbing surface area, and abnormally high top:root ratios (Iyer, 1964; Iyer and Wilde, 1965). The effect of eradicants on the gross morphology of tree seedlings is recorded in figure 1. Figure 2 illustrates the impeded growth of excised roots inflicted by volatile biocide by-products (Persidsky and Wilde, 1954; Iyer, 1968).

As revealed by recent observations, repeated applications of certain fumigants cause a radical alteration of the soil productive capacity by eradicating mycorrhiza-forming fungi and other beneficial organisms of the root zone (Iyer, 1964; Iyer and Wilde, 1965). The exclusion of these essential constituents of mycotrophy precludes the uptake of some nutrients by tree seedlings even from soils which are abundant with these nutrients in readily available form (Voigt, 1955). According to Henderson and Stone (1967), elimination of mycorrhiza-formers by fumigation with methyl bromide, Trizone, and Vapam severely reduced the uptake of phosphorus by coniferous seedlings in New York nurseries. As disclosed by Lipas (1968), repeated treatment of soils with Mylone and Vapam almost completely arrested the uptake of both phosphorus and potassium in some Wisconsin nurseries. The disruption of the mycotrophic mechanism by organic fumigants often does not manifest itself by decreasing plant growth, but by exhibiting foliar symptoms of nutrient deficiencies. Results of soil and plant tissue analyses provide an illustration of a biocide-induced nutritional discrepancy (table 1). Prolonged use of Mylone and Vapam on red pine seedlings markedly increased nitrogen and decreased phosphorus and potassium contents of the foliage.

Improvement of the quality of nursery stock impaired by biocide treatments requires a decrease in the rate of growth and succulence of tree crowns, an increase in absorbing surface area of the roots, and preservation or reintroduction of viable mycorrhiza-forming and extra-matrical fungal mycelia essential for the mycotrophic uptake of nutrients. Among several approaches to the solution of this complicated problem, the most promising results were obtained in trials using the combined effects of fermented sawdust compost and aluminum sulfate in solution.

[1] Contribution from Soil Science Department, University of Wisconsin, in cooperation with and supported in part by the Wisconsin Department of Natural Resources and the W. K. Kellogg Foundation. Publication approved by the Director of the Wisconsin Agr. Exp. Station.

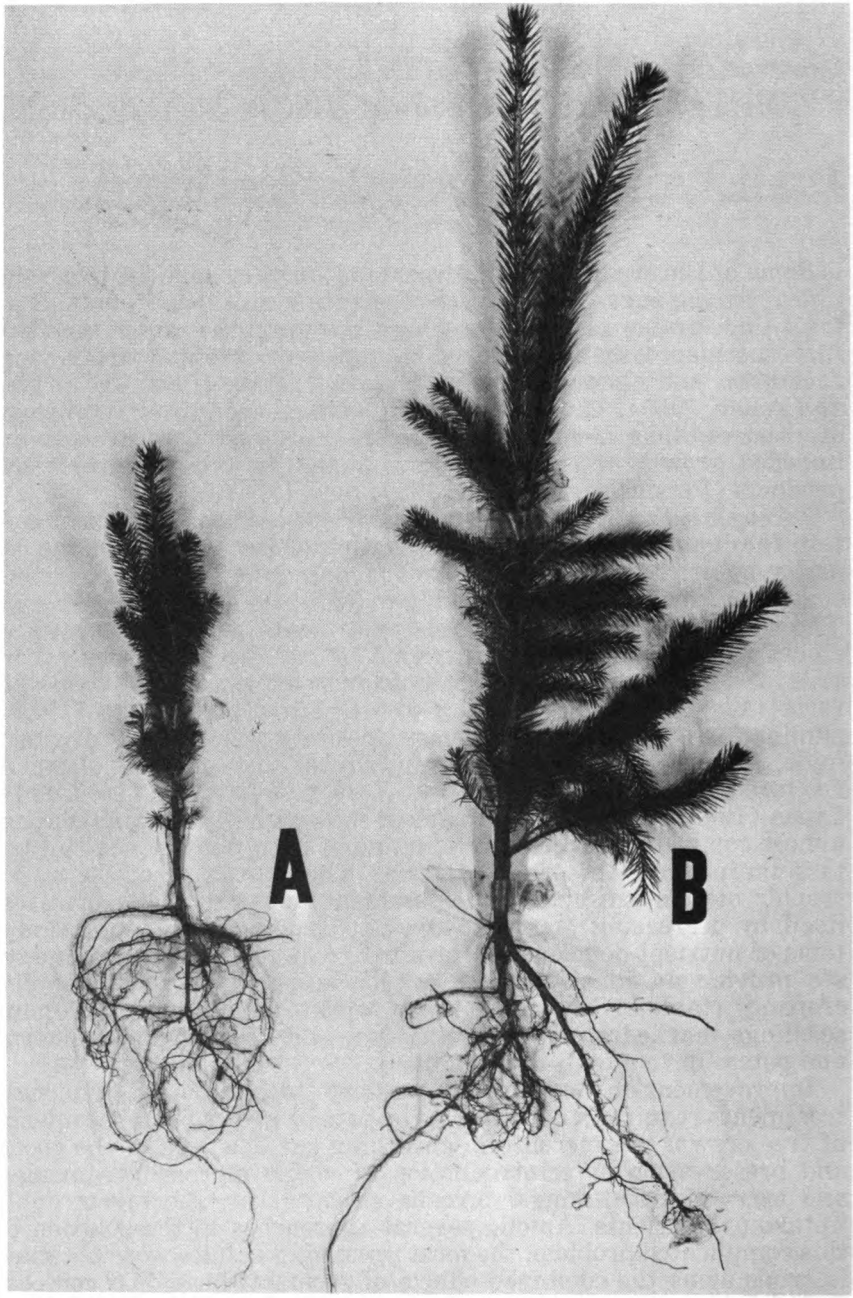

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Figure 1.—Abnormal biocide-induced growth stimulation of 3-year-old white spruce, *Picea glauca*, raised in the following media: A. Untreated nursery soil (Plainfield sand), B. adjacent nursery soil treated with Mylone at a rate of 800 lbs of 50-D and 600 lbs of 85-W per acre. Note the contrast in the development of tops and roots.

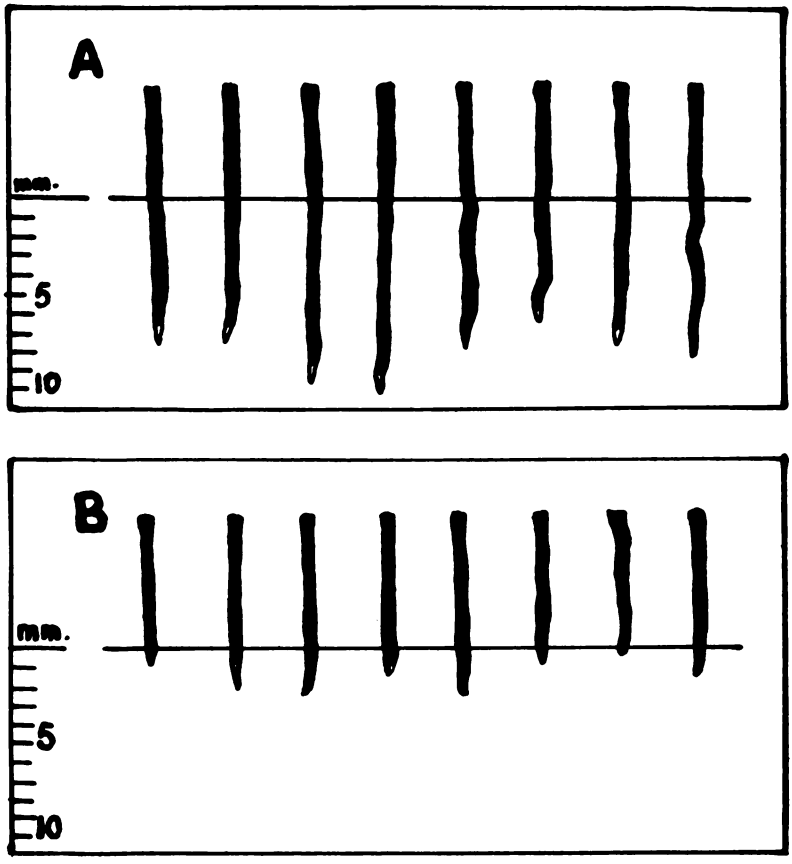

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Figure 2.—Effect of volatile organic substances (CH_3NCS, methylisothiocyanate) released from soils on the growth of excised roots of blue lupine *(Lupinus occidentalis)* after 96 hours of incubation at 23° C. A. Untreated, sandy nursery soil—average root growth 8.1 ± 0.35 mm, B. similar soil treated with Vapam at a rate of 60 pounds per acre—average root growth 2.0 ± 0.70 mm (After Iyer, 1968).

Table 1.—*Soil fertility and uptake of nitrogen, phosphorus and potassium by 1-year-old red pine seedlings from untreated soil and soil subjected to repeated applications of Mylone and Vapam in Hugo Sauer Wisconsin State Nursery (average of four determinations)*

Soil	Soil pH	Total N content of soil. %	Available soil P_2O_5, lb/acre	Available soil K_2O, lb/acre	Exchangeable Ca, me/100g	Exchangeable Mg, me/100g	Percent Foliar content of N	Percent Foliar content of P	Percent Foliar content of K
Untreated	5.5	0.066	282	210	1.25	0.52	1.18	0.21	0.31
Biocide treated	5.7	0.070	268	235	1.42	0.64	1.82	0.09	0.10

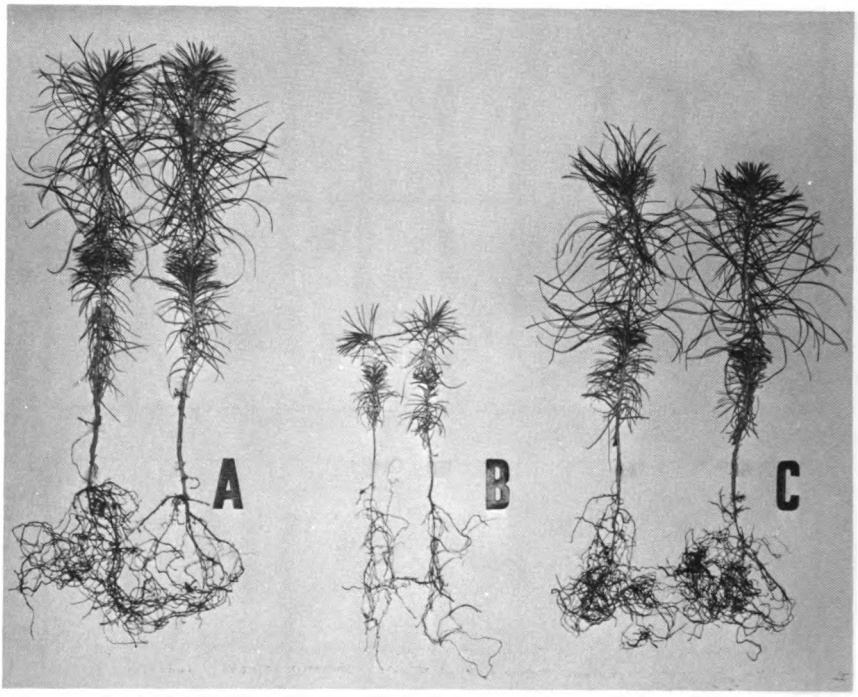

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Figure 3.—Effect of aluminum sulfate and fermented sawdust compost on the morphological features of 1-year-old seedlings of Monterey pine, *Pinus radiata*, raised in a sandy soil receiving the following treatments: A. Mylone (1,400 lbs per acre), B. mylone and aluminum sulfate (800 lbs per acre), C. mylone, aluminum sulfate, and fermented sawdust compost (40 yd³ per acre).

The fermented compost is prepared by treating raw sawdust with anhydrous ammonia, neutralizing the high alkalinity of the product with phosphoric acid and adding potassium sulfate followed by inoculating the mash with *Coprinus ephemerus* (Davey, 1953; Wilde, 1958). Upon completion of fermentation, the compost is sprayed with a humate suspension consisting of an aqueous extract of organic soil layers taken from hardwood-coniferous or coniferous forest stands. Prior to spraying with the humate suspension, the reaction of the suspension is adjusted to pH 5.5 by addition of either 0.2 *N* H_2SO_4 or 0.2 *N* NH_4OH. It appears that symbiotic organisms introduced with humate suspension are protected from the toxic effects of fumigants by a colloidal humate film formed around them and thereby are capable of re-establishing their normal mycotrophic uptake of nutrients. In part, however, the nutrition of seedlings is augmented by the formation of ammonium phosphate during preparation of the fermented compost. This type of phosphate is likely to have greater availability than soil phosphorus extractible with dilute sulfuric or hydrofluoric acids.

Following application of compost, the soil is acidified with aluminum sulfate solution to slightly below pH 4.8. This treatment retards the release of available nitrogen and reduces the foliar growth of the stock.

Aluminum sulfate, $Al_2(SO_4)_3.18H_2O$, is very soluble (87 g per 100 ml). The desired amount of the chemical can be established easily by treatment of small samples of soils and the determination of resulting pH values. Another highly important advantage of aluminum sulfate is that application can be made by hand, with a power sprayer (Brener, 1939), or through an irrigation system.

Table 2 and fig. 3 illustrate the combined effect of compost and aluminum sulfate in greenhouse trials on the growth and morphological characteristics of Monterey pine, *Pinus radiata*. The seedlings were raised in a coarse sandy soil treated with Mylone at a rate of 800 lbs of 50-D and 600 lbs of 85-W per acre, and with Mylone in combination with 40 cubic yards per acre of fermented compost and 800 lbs per acre of aluminum sulfate in the form of a 4 percent solution. The latter treatment appreciably reduced the top:root and height:diameter ratios and significantly increased the specific gravity of the stems and the absorbing capacity (titration value) of the roots of tree seedlings. Improvement in all of these attributes of nursery stock quality shows promise that seedlings treated to alleviate mycotrophic deficiencies in the manner described will be more able to withstand drought, frost, snowpress, and parasitic organisms.

Table 2.—*Morpho-anatomical properties of 1-year-old seedlings of Monterey pine* (Pinus radiata) *raised on nursery beds with combinations of Mylone (800 lbs of 50–D and 600 lbs of 85–W per acre), fermented sawdust compost (40 yd² per acre) and aluminum sulfate (800 lbs per acre) (Results per average seedling)*

Stock properties	Seedlings raised on treated beds		
	Mylone	Mylone and $Al_2(SO_4)_3$	Mylone, $Al_2(SO_4)_3$ and compost
Height, cm	22.0	11.9	19.0
Diameter, mm	1.8	1.4	1.8
Height: diameter ratio	12.2	8.5	10.5
Oven-dry weight, g	0.92	0.50	0.79
Top: root ratio	3.7	1.4	2.7
Specific gravity of stems	0.34	0.47	0.41
Root titration, ml 0.3 *N* NaOH	0.13	0.10	0.49

Summary

Some of the biocidic fumigants such as Trizone, Mylone, and Vapam eradicate mycorrhiza-forming fungi and other beneficial organisms of nursery soils. In consequence, tree planting stock exhibits impeded capacity for the uptake of phosphorus and potassium even when these elements are abundant in the soil in available form.

Re-establishment of the normal nutrition of Monterey pine (*Pinus radiata*) seedlings on Mylone-treated soil was achieved by application of fermented compost sprayed with a humate suspension and by acidification of the soil to slightly below pH 4.8 with a 4% solution of aluminum sulfate. In this treatment the introduced symbiotic microorganisms are protected from the toxicity of eradicants by the organic colloids, whereas acidification retards the release of available nitrogen and reduces foliar growth of the seedlings. Aside from the correction of mycotrophic deficiencies significantly higher survival potential of the nursery stock was observed which was related to decreased succulence, reduced top:root ratio, and increased absorbing surface area of roots.

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Preparation of Mycorrhizal Grain Spawn and Its Practical Feasibility in Artificial Inoculation

J. Y. Park

Many promising results have been reported using spores or mycelial suspensions of mycorrhizal fungi as inoculum with peat moss, duff, or leaf litter as fungus carriers (Hatch 1937, Rayner 1938, McComb 1943, Gilmour 1958, Harley 1959, Briscoe 1959, Bowen 1965, 1966). However, these works are still in the exploratory stage, and are uneconomical on a large scale basis.

In an attempt to establish an effective method of producing large quantities of mycelia for inoculation, the author prepared wheat grain spawn according to a method used in preparation of commercial mushroom spawn. Five grams of wheat grains, 100 mg of calcium carbonate, and 8 ml of water were autoclaved in a test tube for 30 minutes. The tubes were inoculated and incubated for 2 to 3 weeks at 25° C. These cultures were designated the master culture and served as parent inoculum for subsequent inoculations.

In the mass production of grain spawn, the grain was soaked in water, boiled for half an hour, and spread on a dry cloth to remove excess moisture. Four to 5 g of calcium carbonate was mixed with 100 g of grain. The grain was autoclaved in conical flasks at 15-pound pressure for 30 minutes. The autoclaved flasks were inoculated with the master culture, incubated at 25° C and shaken once each week.

A single spawn grain was placed in the middle of a microslide and covered with soil. Another microslide, taped to top of the first, provided a cover for the spawn and soil. This was incubated at 20°C in a stain-jar filled with soil and water. After a few days the the advancement of hyphae was determined under a stereomicroscope.

The hyphae were removed from the flask and fragmented in a Waring blendor. Hyphal growth was determined quantitatively by the method of Jones and Mollison (1948). Mycelial fragments were counted using Petroff-Hausser bacteria counter and an eyepiece micrometer, and the number of mycelial fragments was multiplied by the average length of individual hyphal fragments in order to get the total length of the hyphal fragments.

Adopting the foregoing methods, grain, leaf litter, and peat moss spawn of *Suillus granulatus* and *Cenococcum graniforme* were prepared. Hyphae production and growth rate with the grain spawn were always five to eight times higher than with the other two spawns. It was found that a few grains per planting hole and 2 liters of grain spawn per acre would be sufficient for inoculating the mycorrhizal fungi mentioned. Grain spawn may be an ideal substance for artificial inoculation of mycorrhizal fungi into the soil.

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Separate No. FS–307

Mycorrhiza Studies in Manitoba and Saskatchewan

R. D. Whitney

Studies on Polyporus tomentosus

Gosselin (1944) suggested that *Polyporus tomentosus* Fr. formed mycorrhizae with spruce of eastern Canada; however, in studies of white spruce (*Picea glauca* (Moench) Voss) in Manitoba and Saskatchewan, this fungus was identified as the main causative agent of root and butt-rot. Subsequent laboratory studies were initiated to determine the mycorrhiza forming potential of *P. tomentosus*.

In recent studies (Whitney, 1962, 1965), *P. tomentosus* was inoculated into flasks containing aseptically grown white spruce seedlings. Mycelia grew readily throughout the medium, forming a dense crust-like layer on the roots. Although hyphal invasion of the cortical cells was extensive, there was no suggestion of Hartig net formation. At first, many of the plants appeared healthy, but all eventually died. In those white spruce seedlings inoculated with natural mycorrhizal tips, ectomycorrhizal formation was confirmed.

Further tests were conducted using seedlings of red and black spruce (*Picea rubens* Sarg. and *P. mariana* (Mill) B.S.P.). After 12 months' exposure to 10 isolates of *P. tomentosus and P. tomentosus*, variety *circinatus* Fr., mycorrhizae still did not develop. The effect of the fungus was essentially the same as that on white spruce, although the pathogenic effects of the isolates varied.

There is little if any toxic effect resulting from this fungus. Death of the seedlings was probably caused by mechanical disruption of the conducting system. All spruce species tested appeared to have a wide tolerance to the invasion of *P. tomentosus* and the variety *circinatus*; however, a symbiosis was not induced.

Attempts were also made to isolate *P. tomentosus* from mycorrhizae of trees in plots where the fungus was known to be present (Whitney, 1962). Hundreds of sterilized mycorrhizal tips yielded several fungi suspected of forming mycorrhizae. *P. tomentosus* was not one of them.

Studies to Improve Mycorrhiza Development

Red pine, jack pine, Scots pine, and white spruce from the Hadashville nursery in Manitoba invariably have been found to contain mycorrhizae. However, mycorrhiza development is poor in all species up to three years of age. The application of commercial fertilizer in various quantities and combinations did not alter mycorrhiza formation in one-year-old conifers. There were no anatomical or physical differences between the fertilized and unfertilized

mycorrhizal seedlings. In all cases, mycorrhiza development was measured by the percentage of infected roots, the depth of the Hartig net, and the completion of the hyphal mantle.

Studies on Effects of Burning

Burning for silvicultural purposes is in the experimental stage in Manitoba and Saskatchewan. As a part of a larger investigation of the establishment, survival and growth of seedlings following burning, a study of mycorrhiza development on sites exposed to various burn intensities is being conducted.

Studies of Mycorrhiza Formation in Various Soils

Mycorrhizal studies have been initiated in the agricultural zone of Manitoba and Saskatchewan. It is suspected that the decline and death of trees in certain areas is due to the absence of appropriate mycorrhizal fungi in the soils. Whether this is caused by unfavorable soil conditions such as alkalinity, salinity, or drought or by the distance of these areas from mycorrhizal symbionts is now being determined.

A group of nonmycorrhizal seedlings will be planted in various types of soils throughout the region and periodically, their roots will be examined for mycorrhizae. The suitability of conditions for mycorrhiza development will be determined with artificial inoculations.

Another group of nonmycorrhizal seedlings will be planted in the greenhouse on soil obtained from each of these areas. After appropriate inoculations, roots will be examined for mycorrhiza development.

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Conference Participants

DAVALINE T. BESLOW, USDA Forest Service, Beltsville, Md.
W. M. BEVER, University of Illinois, Urbana
M. VICTOR BILAN, Stephen F. Austin State College, Nacogdoches, Tex.
JAMES R. BOYLE, University of Wisconsin, Madison
L. E. CASIDA, JR., The Pennsylvania State University, University Park
E. E. C. CLEBSCH, University of Tennessee, Knoxville
DARRELL COX, University of Illinois, Urbana
C. B. DAVEY, North Carolina State University, Raleigh
RICHARD E. DICKSON, USDA Forest Service, Ames, Iowa
K. D. DOAK, Hebron, Ind.
M. V. DUBLIN, Urbana, Ill.
HUGO FERCHAU, Western State College, Gunnison, Col.
J. ANDRE FORTIN, Université Laval, Quebec, Canada
THOMAS E. FURMAN, University of New Hampshire, Durham
J. W. GERDEMANN, University of Illinois, Urbana
LARRY F. GRAND, North Carolina State University, Raleigh
LYNN E. GRAY, USDA Agricultural Research Service, Urbana, Ill.
EDWARD HACSKAYLO, USDA Forest Service, Beltsville, Md.
A. B. HATCH, Peterboro, N. Y.
VIRGIL K. HOWE, Western Illinois University, Macomb
JAYA G. IYER, University of Wisconsin, Madison
KENNETH J. KESSLER, USDA Forest Service, Marquette, Mich.
GARY D. KLEINSCHMIDT, University of Illinois, Urbana
GEORGE N. KRYWOLAP, University of Maryland, Baltimore
ROBERT F. LUCAS, Cornell University, Ithaca, N. Y.
DONALD H. MARX, USDA Forest Service, Athens, Ga.
CARLOS O. MILLER, Indiana University, Bloomington
ORSON K. MILLER, USDA Forest Service, Laurel, Md.
HAROLD L. MITCHELL, USDA Forest Service, Madison, Wis.
CAROLYN NAPIER, Northwestern State College, Natchitoches, La.
J. Y. PARK, Canada Department of Forestry and Rural Development, Sault Ste. Marie
J. G. PALMER, USDA Forest Service, Laurel, Md.
RONALD H. PETERSEN, University of Tennessee, Knoxville
C. P. P. REID, Colorado State University, Fort Collins
JERRY W. RIFFLE, USDA Forest Service, Albuquerque, N. Mex.
D. P. ROGERS, University of Illinois, Urbana
G. SAFIR, University of Illinois, Urbana
THOMAS SANTORO, Brooklyn Botanic Garden, Ossining, N. Y.
V. ŠAŠEK, Iowa State University, Ames, (now Inst. of Miciobiol., Prague, Czechoslovakia)
JEAN SCHOKNECHT, University of Illinois, Urbana
W. A. SINCLAIR, Cornell University, Ithaca, N. Y.
ROLF SINGER, Field Museum of Natural History, Chicago, Ill.
MRS. ROLF SINGER, Chicago, Ill.
V. SLANKIS, Canada Department of Forestry and Rural Development, Maple, Ontario

ALEXANDER H. SMITH, University of Michigan, Ann Arbor
N. M. STARK, University of Nevada, Reno
EARL M. STONE, Cornell University, Ithaca, N. Y.
JAMES M. TRAPPE, USDA Forest Service, Corvallis, Oreg.
G. K. VOIGT, Yale University, New Haven, Conn.
J. A. VOZZO, USDA Forest Service, Houston, Tex.
F. W. WENT, University of Nevada, Reno
R. D. WHITNEY, Canada Department of Forestry and Rural Development, Winnipeg
HUGH WILCOX, College of Forestry, Syracuse, N. Y.
S. A. WILDE, University of Wisconsin, Madison
E. BURWELL WINGFIELD, Virginia Military Institute, Lexington
B. ZAK, USDA Forest Service, Corvalis, Oreg.

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Organism Index

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